PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K A2

(11) International Publication Number: **WO 00/03683**

(43) International Publication Date: 27 January 2000 (27.01.00)

(21) International Application Number: PCT/US99/16166

(22) International Filing Date: 16 July 1999 (16.07.99)

(30) Priority Data:
60/093,481 20 July 1998 (20.07.98) US

(71) Applicant *(for all designated States except US)*: INEX PHARMACEUTICALS CORPORATION [US/US]; Glenyon Business Park, 100–8900 Glenlyon Parkway, Burnaby, British Columbia V6K 3S4 (CA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BOEY, Anthony [CA/CA]; 419–2551 Parkview Lane, Port Coquitlam, British Columbia V3C 6J8 (CA). CHEN, Tao [CA/CA]; 9–7711 Minorn Boulevard, Richmond, British Columbia V64 1Z3 (CA). LENG, Esther [CA/CA]; 14136 88th Avenue, Surrey, British Columbia V3W 3L6 (CA). TARD, Paul, G. [CA/CA]; 19081 Sundale Court, Surrey, British Columbia V3S 7M6 (CA). WHEELER, Jeffrey, J. [US/CA]; 6179 17A Street, Surrey, British Columbia V34 8G9 (CA). SCHERRER, Peter [CH/CA]; 2664 Birch, Vancouver, British Columbia V6H 2T5 (CA). GRAHAM, Roger [CA/CA]; 2638 West 7th Avenue, Vancouver, British Columbia V6K 1Z1 (CA).

(74) Agents: SNYDER, Joseph, R. et al.; Townsend and Townsend and Crew LLP, Two Embarcadero Center, 8th floor, San Francisco, CA 94111–3834 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: LIPOSOMAL ENCAPSULATED NUCLEIC ACID–COMPLEXES

(57) Abstract

This invention relates to liposomes which are useful for the introduction of nucleic acids into cells. The liposomes of the present invention entrap a condensing agent–nucleic acid complex and are suitable for nucleic acid–transfer delivery vehicles in clinical use. In addition, methods of transfecting a cell with a nucleic acid using the liposomes of the present invention are also disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

LIPOSOMAL ENCAPSULATED NUCLEIC ACID-COMPLEXES

FIELD OF THE INVENTION

This invention relates to liposomes that are useful for introducing nucleic acids into cells. More particularly, the liposomes of the present invention entrap a condensing agent-nucleic acid complex and, thus, they are useful as nucleic acid-transfer delivery vehicles in clinical use.

BACKGROUND OF THE INVENTION

The introduction of foreign genes and other molecules into cells is of great interest to molecular biologists. One reason to introduce genetic material into cells is to express an encoded protein. Gene transfer involves delivering nucleic acids to target cells and then transferring the nucleic acid across the cell membrane in a form that can function in a therapeutic manner. Of the many methods used to facilitate entry of DNA into eukaryotic cells, liposomes are among the most efficacious and have found extensive use as DNA carriers in transfection experiments. Cationic lipids are known to bind to polynucleotides and to facilitate their intracellular delivery into mammalian cells. Nucleic acid is negatively charged and when combined with a positively charged lipid, forms a complex that is suitable for formulation and cellular delivery. The use of cationic lipid carriers for transfection is well established.

Other gene transfer methods under study include viral vectors. Although viral vectors have the inherent ability to transport nucleic acids across cell membranes and, in some instances, integrate exogenous DNA into chromosomes, they can carry only limited amounts of DNA and pose several risks. One such risk involves the random integration of viral genetic sequences into patient chromosomes, potentially damaging the genome and possibly inducing a malignant transformation. Another risk is that the viral vector may revert to a pathogenic genotype either through mutation or genetic exchange with a wild type virus.

Limitations associated with viral gene delivery systems have spawned the development of nonviral gene transfer vectors. These nonviral systems generally consist of plasmid DNA complexed to a cationic agent, such as a lipid or polymer, to condense the nucleic acid and to facilitate its cellular uptake into the cell membrane. One of the obstacles to gene expression is the degradation of the DNA in route to the nucleus within

the cytoplasm. In this respect, polycations have been used extensively to overcome this obstacle and improve gene expression. These cationic polymers include antibiotics, such as gramicidin S, dendrimers or cascade polymers or cationically modified albumin. In addition, spermidine has been shown to condense DNA and improve transfection of cell cultures. These condensing agents protect the DNA from degradation by endonucleases and restriction enzymes. The positive charge on these polymers is also expected to boost the transfection capability of the complexes.

Other polycationic polymers that are useful as condensing agents because of their affinity to electrostatically bind nucleic acids include polylysine, polyarginine and polyornithine. The polycation polyethylenimine (PEI), which is a highly-branched polymer, has been shown to be a highly efficient gene delivery agent. In this regard, PEI condenses nucleic acid into a highly compact form and offers good protection from various nucleases. It has been reported that the gene transfer with these complexes was boosted up to a 1000-fold, under certain conditions. Clearly, polycations like PEI have a clear advantage over the lipid/nucleic acid complexes in this respect.

However, one significant drawback of polycationic-nucleic acid complexes, such as a PEI-nucleic acid complex, is the toxicity associated with *in vivo* gene delivery via the use of such complexes. When PEI is condensed with nucleic acid at higher ratios, the complexes become toxic. At lower ratios (~2), transfection is reduced significantly. If these highly transfecting particles are to be used *in vivo* for transfection, the toxicity must be reduced to tolerable levels.

As such, there exists a need to design condensing agent-nucleic acid complexes that are effective for facilitating intracellular delivery of genetic material, but that will reduce the associated cellular toxicity. The present invention fulfills this and other needs.

SUMMARY OF THE INVENTION

In one aspect, the present invention relates to a liposome having (a) a lipid; and (b) an encapsulated condensing agent-nucleic acid complex. In certain preferred aspects, the liposomes of the present invention further comprise (c) a bilayer stabilizing component. The bilayer stabilizing component can be reversibly associated with the liposome. Such liposomes are extremely advantageous because they offer good protection to the nucleic acid from various nucleases that tend to degrade nucleic acid that is not protected by encapsulation. Moreover, in many instances, gene transfer with these complexes is increased up to a 1000-fold. In addition, using the encapsulating formulations of the present invention the toxicity of the condensing agents are reduced to tolerable levels.

Condensing agents suitable for use in the present invention include, but are not limited to, polycationic polymers, such as polyethylenimine, polylysine, polyarginine and polyornithine. Other condensing agents that have an affinity for nucleic acid and that are suitable for use in the present invention include, but are not limited to, natural DNA-binding proteins of a polycationic nature, such as histones and protamines or analogues or fragments thereof. Other condensing agents suitable for use in the present invention include spermidine, spermine, polycations having two or more different positively charged amino acids or basic proteins.

Although numerous lipids can be used, the lipids used in the liposomes of the present invention are preferably non-cationic lipids. Such non-cationic lipids include, but are not limited to, ceramides, phosphatidylethanolamines, phosphatidylserines and mixtures thereof. In a presently preferred embodiment, the non-cationic lipids used are ceramides, dioleoylphosphatidylethanolamine, dioleoylphosphatidylserine and mixtures thereof.

In another aspect, the present invention relates to a method for encapsulating a condensing agent-nucleic acid complex in a liposome, the method comprising: adding a condensing agent solution into a nucleic acid solution to form a condensing agent-nucleic acid complex; and adding said condensing agent-nucleic acid complex to a lipid suspension to form an encapsulated condensing agent-nucleic acid complex. In a preferred embodiment, the method comprises:

(a) admixing a first condensing agent solution into a nucleic acid solution to form precondensed nucleic acid;

(b) adding the precondensed nucleic acid into a second condensing agent solution to form a condensing agent-nucleic acid complex;

(c) dialyzing the condensing agent-nucleic acid complex to form a concentrated condensing agent-nucleic acid complex;

(d) adding the concentrated condensing agent-nucleic acid complex to a lipid suspension containing detergent; and

(e) removing the detergent from the lipid suspension to form an encapsulated condensing agent-nucleic acid complex in the liposome.

In this method, the first and second condensing agents can be the same or different.

In yet another aspect, the present invention relates to a method of transfecting a cell with a nucleic acid, the method comprises contacting the cell with a liposome having (a) a lipid; and (b) an encapsulated condensing agent-nucleic acid complex. In certain preferred embodiments, the liposomes of this method further comprise (c) a bilayer stabilizing component. The bilayer stabilizing component can be reversibly associated with the liposome.

In still yet another aspect, this invention relates to the treatment of a disease involving the transfection of a cell with nucleic acid and the introduction into cells of antisense nucleotides, as well as the stable transfection of a cell with DNA engineered to become incorporated into the genome of the living cell.

Other features, objects and advantages of the invention and its preferred embodiments will become apparent from the detailed description which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the construction of uniformed small size polyethylenimine-nucleic acid complex.

Figure 2 illustrates an effect of dextran sulfate on PEI-DNA complexes.

Figure 3 illustrates the titration of dextran sulfate to determine the minimal amount of dextran sulfate required to completely expose DNA to picogreen.

Figure 4 illustrates a standard curve for quantifying DNA. At each data point, a standard amount of dextran sulfate is added, which is the same amount added to the random test samples of complexes.

Figure 5 illustrates that the relaxation or dissociation of the complexes is not an instantaneous event.

Figure 6 illustrates the extent of encapsulation when dextran sulfate is used in the assay to dissociate the nucleic acid from the PEI.

Figure 7 illustrates a titration of DOPS to optimize encapsulation efficiency.

Figure 8 illustrates a Guassian size distribution of a sample of lipid encapsulated PEI/DNA containing 8 mol% DOPS. The liposomes are typically around 75 to about 80 nm in diameter.

Figure 9 illustrates transfection of Cos-7 cells with encapsulated PEI condensed DNA liposomes-dose response and time course.

Figure 10 illustrates transfection of various cell lines with encapsulated PEI condensed DNA Liposomes. LS-180 is derived from a 58 year old female patient with the Duke's type adenocarcinoma of the colon; SK-OV-3 is a human ovarian adenocarcinoma tumor taken from a 64 year old; U87 is human glioblastoma; COS-7 is kidney, fibroblast-like cell line established from CV-1 simian cells which were transformed by an origin-defective mutant of SV40; Lewis Lung is human lung carcinoma; and B16 is mouse melanoma.

Figure 11 illustrates an *in vitro* toxicity of encapsulated PEI condensed DNA liposomes in Cos-7 cell line.

Figure 12 illustrates concentration dependence of the condensing agent-nucleic acid complex on cell death.

Figure 13 illustrates *in vivo* gene expression of PEI condensed DNA liposomes in Lewis Lung tumor.

Figure 14 illustrates gene expression of encapsulated PEI condensed DNA in B16 i.p. tumor.

Figure 15 illustrates *in vitro* toxicity of pre and post purification of encapsulated PEI condensed DNA liposomes.

DESCRIPTION OF THE PREFERRED EMBODIMENT

A. Glossary

The term "lipid" refers to any suitable material resulting in a bilayer such that a hydrophobic portion of the lipid material orients toward the bilayer while a hydrophilic portion orients toward the aqueous phase. Amphipathic lipids have a hydrophilic portion and a hydrophobic portion. Hydrophilic characteristics derive from the presence of phosphato, carboxylic, sulfato, amino, sulfhydryl, nitro, and other like

groups. Hydrophobicity could be conferred by the inclusion of groups that include, but are not limited to, long chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic or heterocyclic group(s). Amphipathic compounds include, but are not limited to, phosphoglycerides and sphingolipids, representative examples of which include phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine or dilinoleoylphosphatidylcholine. Other compounds lacking in phosphorus, such as sphingolipid and glycosphingolipid families are also within the group designated as lipid. Additionally, the amphipathic lipids described above may be mixed with other lipids including triglycerides and sterols.

The term "neutral lipid" refers to any of a number of lipid species which exist either in an uncharged or neutral zwitterionic form at physiological pH. Such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, and cerebrosides.

The term "non-cationic lipid" refers to any neutral lipid as described above as well as anionic lipids. Preferred non-cationic lipids include phosphatidylethanolamines, phosphatidylserines and ceramides. Examples of preferred anionic lipids include cardiolipin, diacylphosphatidylserine, diacylphosphatidic acid, N-succinyl-phosphatydylethanolamine (N-succinyl-PE), phosphatidic acid, phosphatidylinositol, phosphatidylglycerol and phosphatidyl ethylene glycol.

The term "cationic lipid" refers to any of a number of lipid species which carry a net positive charge at physiological pH. Such lipids include, but are not limited to, DODAC, DOTMA, DDAB, DOTAP, DC-Chol and DMRIE. Additionally, there are a number of commercial preparations of cationic lipids. These include, for example, LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, New York, USA); LIPOFECTAMINE® (commercially available cationic liposomes comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic lipids comprising DOGS in ethanol from Promega Corp., Madison, Wisconsin, USA).

The term "bilayer stabilizing component" as used herein refers to compounds (*e.g.*, lipids, polymers, *etc.*) that allow lipids adopting a non-lamellar phase under physiological conditions to be stabilized in a bilayer structure. The bilayer

stabilizing components are either bilayer forming themselves, or are of a complementary dynamic shape. The non-bilayer forming lipid is stabilized in the bilayer structure when it is associated with, *i.e.*, in the presence of, the bilayer stabilizing component. In certain embodiments the bilayer stabilizing component is capable of transferring out of the liposome, or of being chemically modified by endogenous systems such that, with time, it loses its ability to stabilize the lipid in a bilayer structure. When liposomal stability is lost, destabilized or decreased, fusion can occur. Fusion can result in the release of liposome payload into the target cell. Thus, in certain embodiments, the bilayer stabilizing component is, "reversibly associated" with the lipid and when it is associated with the lipid, the lipid is constrained to adopt the bilayer structure under conditions where it would otherwise adopt a non-lamellar phase. As such, the bilayer stabilizing components of the present invention is capable of stabilizing the lipid in a bilayer structure, yet is capable of exchanging out of the liposome, or of being chemically modified by endogenous systems so that, with time, they lose their ability to stabilize the lipid in a bilayer structure, thereby allowing the liposome to become fusogenic or release its payload.

In certain other embodiments, the bilayer stabilizing component does not transfer out of the liposome. In these embodiments, the liposome is non-fusogenic and the bilayer stabilizing component is not, "reversibly associated" with the lipid.

The term "transfection" as used herein, refers to the introduction of polyanionic materials, particularly nucleic acids, into cells. The term "lipofection" refers to the introduction of such materials in association with lipids. The polyanionic materials can be in the form of DNA or RNA which is linked to expression vectors to facilitate gene expression after entry into the cell. Thus, the polyanionic material used in the present invention is meant to include DNA having coding sequences for structural proteins, receptors and hormones, as well as transcriptional and translational regulatory elements (*i.e.*, promoters, enhancers, terminators and signal sequences) and vector sequences. Methods of incorporating particular nucleic acids into expression vectors are well known to those of skill in the art, but are described in detail in, for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) or *Current Protocols in Molecular Biology*, F. Ausubel *et al.*, ed. Greene Publishing and Wiley-Interscience, New York (1987), both of which are incorporated herein by reference.

"Expression vectors," "cloning vectors," or "vectors" are often plasmids or other nucleic acid molecules that are able to replicate in a chosen host cell. Expression vectors may replicate autonomously, or they may replicate by being inserted into the genome of the host cell, by methods well known in the art. Vectors that replicate autonomously will have an origin of replication or autonomous replicating sequence (ARS) that is functional in the chosen host cell(s). Often, it is desirable for a vector to be usable in more than one host cell, *e.g.*, in *E. coli* for cloning and construction, and in a mammalian cell for expression.

The term "encapsulation" as used herein when discussing amount of encapsulation, refers to the amount of condensing agent-nucleic acid complex that is unavailable to picogreen binding in a picogreen/dextran binding assay or that is nuclease resistant in a nuclease assay.

B. General

It has now been discovered that lipid encapsulation of a condensing agent-nucleic acid complex offers greater protection against enzymatic digestion and gives consistently higher gene expression than unencapsulated condensing agent-nucleic acid complexes. As such, in one aspect, the present invention relates to a liposome comprising (a) a lipid; and (b) an encapsulated condensing agent-nucleic acid complex. In certain preferred embodiments, the liposome further comprises (c) a bilayer stabilizing component. The bilayer stabilizing component can be reversibly associated with the liposome.

The condensing agents used in the liposomes of the present invention can be any compound that has the ability to complex and compact nucleic acids. The complex generally comprises at least one negatively charged nucleic acid and at least one positively charged polymer, the association between the nucleic acid and the cationic polymer being electrostatic in nature.

As such, the condensing agents suitable for use in the present invention include, but are not limited to, polycationic polymers, such as polyethylenimine, polylysine, polyarginine and polyornithine. Other condensing agents that have an affinity for nucleic acid and that are suitable for use in the present invention include, but are not limited to, natural DNA-binding proteins of a polycationic nature, such as histones and protamines or analogues or fragments thereof. Other condensing agents suitable for use in the present invention include polyamines including, but not limited to, spermidine and

spermine, polycations having two or more different positively charged amino acids or basic proteins. In a preferred embodiment, the condensing agent is a polycationic polymer. In another preferred embodiment, condensing agents other than cationic lipids are used. Those of skill in the art will be aware of other condensing agents suitable for use in the present invention.

A particularly preferred example of a polycationic polymer is polyethylenimine. Polyethylenimine, which is a polymeric substance wherein every third atom is an amino nitrogen that can be protonated, has the general formula:

$$(\text{-NH-CH}_2\ \text{CH}_2\text{-})_x[\text{-N}(\text{CH}_2\text{CH}_2\text{NH}_2)\text{CH}_2\text{CH}_2\text{-}]_y \qquad \text{I}$$

In formula I, x is approximately 2 times the value of y. Polyethylenimine is a highly branched material where the ratio of primary to secondary to tertiary nitrogens is about 1:2:1. The primary nitrogens equal the tertiary nitrogens because each branch point has a chain end. Polyethylenimine of various molecular weights can be used. Preferably, molecular weights between 0.8 kDa to about 800 kDa can be used. More preferably, a molecular weight of about 25 kDa can be used. It will be apparent to those of skill in the art that various molecular weight polymers of polyethylenimine will be suitable for use in the present invention. Polyethylenimine of various molecular weights is commercially available from Aldrich Chemical Co., (Milwaukee, Wisconsin).

The nucleic acids of this invention are typically nucleotide polymers having from 10 to 100,000 nucleotide monomers. The nucleic acids are administered to a subject for the purpose of repairing or enhancing the expression of a cellular protein. Additionally, the nucleic acid can carry a label, *e.g.*, radioactive label, fluorescent label or colorimetric label for the purpose of providing clinical diagnosis relating to the presence or absence of complementary nucleic acids. Accordingly, the nucleic acids, or nucleotide polymers, can be polymers of nucleic acids including genomic DNA, cDNA, mRNA or oligonucleotides containing nucleic acid analogs, for example, the antisense derivatives described in a review by Stein, *et al.*, *Science*, **261**:1004-1011 (1993) and in U.S. Patent Nos. 5,264,423 and 5,276,019, the disclosures of which are incorporated herein by reference. Still further, the nucleic acids can encode transcriptional and translational regulatory sequences including promoter sequences and enhancer sequences.

The nucleotide polymers can be single-stranded DNA or RNA, or double-stranded DNA or DNA-RNA hybrids. In addition, nucleic acid with chemically modified phosphodiester bonds (*e.g.*, thiophosphodiester) are also suitable. Examples of double-

stranded DNA include structural genes, genes including control and termination regions, and self-replicating systems, such as plasmid DNA. In preferred embodiments, the nucleic acid is a plasmid.

Single-stranded nucleic acids include antisense oligonucleotides (complementary to DNA and RNA), ribozymes and triplex-forming oligonucleotides and oligonucleotides having modified chemical backbones. These modifications will preferably have some or all of the nucleotide linkages substituted with stable, non-phosphodiester linkages, including, but not limited to, phosphorothioate, phosphorodithioate, phosphoroselenate, or O-alkyl phosphotriester linkages.

The nucleic acids used in the present invention will also include those nucleic acids in which modifications have been made in one or more sugar moieties and/or in one or more of the pyrimidine or purine bases. Examples of sugar modifications include replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, azido groups or functionalized as ethers or esters. Additionally, the entire sugar may be replaced with sterically and electronically similar structures, including aza-sugars and carbocyclic sugar analogs. Modifications in the purine or pyrimidine base moiety include, for example, alkylated purines and pyrimidines, acylated purines or pyrimidines, or other heterocyclic substitutes known to those of skill in the art.

Multiple genetic sequences can be also be used in the present methods. Thus, the sequences for different proteins may be located on one strand or plasmid. Promoter, enhancer, stress or chemically-regulated promoters, antibiotic-sensitive or nutrient-sensitive regions, as well as therapeutic protein encoding sequences, may be included as required. Non-encoding sequences may be also be present, to the extent that they are necessary to achieve appropriate expression.

The nucleic acids used in the present method can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries or prepared by synthetic methods. Synthetic nucleic acids can be prepared by a variety of solution or solid phase methods. Generally, solid phase synthesis is preferred. Detailed descriptions of the procedures for solid phase synthesis of nucleic acids by phosphite-triester, phosphotriester, and H-phosphonate chemistries are widely available. *See*, for example, Itakura, U.S. Pat. No. 4,401,796; Caruthers *et al.*, U.S. Pat. Nos. 4,458,066 and 4,500,707; Beaucage *et al.*, *Tetrahedron Lett.* **22**:1859-1862 (1981); Matteucci *et al.*, *J. Am. Chem. Soc.* **103**:3185-3191 (1981); Caruthers *et al.*, *Genetic Engineering* **4**:1-17 (1982); Jones, chapter 2, Atkinson *et al.*, chapter 3, and Sproat *et al.*, chapter 4, in

Oligonucleotide Synthesis: A Practical Approach, Gait (ed.), IRL Press, Washington, D.C. (1984); Froehler *et al., Tetrahedron Lett.* **27**:469-472 (1986); Froehler *et al., Nucleic Acids Res.*, **14**:5399-5407 (1986); Sinha *et al. Tetrahedron Lett.* **24**:5843-5846 (1983); and Sinha *et al., Nucl. Acids Res.*, **12**:4539-4557 (1984), which are incorporated herein by reference.

In addition, the nucleic acids of this invention can be chosen from among the following:

(a) gene markers, such as luciferase gene, β-galactosidase gene, chloramphenicol acetyl transferase gene, genes bestowing the resistance to an antibiotic, such as hygromycin or neomycin;

(b) genes for therapeutic purposes, such as gene encoding low density lipoprotein receptors, deficient in the case of hypercholesterolomia (liver), coagulation factors: factors VII and IX, phenylalanine-hydroxylase (phenylketonuria), adenosine deaminase (ADA immunodeficiency), lysosomic enzymes, such as β-glucosidase in the case of Gaucher's disease, dystrophine and minidistriphine (myopathy), tyrosine hydroxylase (Parkinson), neuron growth factors (Alzheimer), CFTR cystic fibrosis transmembrane conductance regulator (mucoviscidose), alpha1-antitrypsin, nuclear factors: NF-KB, CII TA, cytokines and interleukines, TNF: tumor necrosis factor, thymidine kinase of the Herpes simplex virus, NO synthase, angiotensin II receptors, gene suppressors of tumors, such as the gene for the p53 protein, MHC proteins, major histocompatibility system, in particular HLA-B7, antioncogenes: p53, RB, cytosine desaminase, sense and antisense RNA; and

(c) genes with vaccine purposes: genes encoding viral antigens, for example, the nucleoprotein of the influenza virus.

Other suitable nucleic acid for use in the present invention will be readily apparent to those of skill in the art.

C. Preparation of the Condensing Agent-Nucleic Acid Complex

Nucleic acid condensation by polycations is a function of the nature and concentration of all ions present in the condensing media. The complexation is dependent, therefore, on pH, volume and salt concentration of the complexation medium. Cationic polymer condensation with the negatively charged nucleic acid is a cooperative process that can be modulated and even inhibited in high salt concentration.

In addition, it was previously noted in O. Boussif *et al., Proc. Natl. Acad. Sci.* **92**:7297-7301 (1995), incorporated herein by reference, that for the cationic polymer polyethylenimine, the order of adding reagents influences the properties of the resulting particles. For instance, adding the PEI solution dropwise to the nucleic acid, *e.g.*, DNA, solution was 10-fold more efficient than adding nucleic acid to PEI.

The following method illustrates the preparation of a stock cationic polymeric solution using PEI as an example. The cationic polymer, *e.g.*, PEI, is dissolved in deionized water and neutralized to pH 7.4 with, for example, HCl. The neutralized solution is then filtered using a Millipore filter having a pore size of about 0.2 μm. In order to encapsulate the PEI/nucleic acid complex in a liposome (described hereinbelow), a small uniform particle size is critical. Large and heterogenic aggregates are a result of complexation of PEI and nucleic acid using either too high a nucleic acid concentration or solutions other than water.

An important criteria for the condensing agent-nucleic acid complex is the calculation of charge ratio. In one embodiment, the condensing agent-nucleic acid complex bears a net positive charge. A condensing agent-nucleic acid charge ratio of about 10:1 to about 2:1 is preferred, and a ratio of about 7:1 to about 4:1 is more preferred. For the PEI-nucleic acid complexes of this invention, an average mass per negative charge ratio of 325 dalton was used for plasmid DNA. The mass per positive charge for PEI was calculated to be 258 dalton. This assumes that one out of six PEI nitrogens is protonated under physiological conditions, and that the average mass per -CH_2CH_2NH- repeat nitrogen unit in PEI is 43.

In another embodiment, the condensing agent-nucleic acid complex is neutral. In this embodiment, the positive charge of the condensing agent is equal to the negative charge of the nucleic acid. This results in a neutral complex.

Thus, in another embodiment, the present invention relates to a method of condensing a nucleic acid with a condensing agent to give uniformed complexes having a typical size of between about 30 nm to about 60 nm. Using PEI as a typical cationic polymer, the method involves, first, precondensing the nucleic acid by the dropwise addition of a PEI solution (10 μg of stock PEI in 250 mL water) into a nucleic acid solution (100 μg/250 mL) while vortexing. Second, precondensed nucleic acid is saturated with excess PEI. Next, the PEI/nucleic acid complexes are concentrated by dialysis. Finally, the concentrated PEI-nucleic acid complexes are dialyzed overnight against HBS buffer to adjust the salt concentration to 150 mM. In the final step, other

buffers can be used. These buffers include, but are not limited to, PBS, sucrose, water or organic solvent in ethanol, with the ethanol not exceeding 60-70%.

The polyethylenimine:nucleic acid ratio in the complex is about 10:1 wt/wt to about 1.5:1 wt/wt, preferably about 6:1 wt/wt to about 1.5:1 wt/wt, and, more preferably, about 4:1 wt/wt.

It is possible to quantitate the amount of nucleic acid condensed to the polycationic polymer. For instance, at a PEI:nucleic acid wt/wt ratio of 4:1, the complex is tightly condensed and not readily accessible to nucleic acid quantification probes like picogreen. If the nucleic acid is free and not complexed to the condensing agent, picogreen will bind to the nucleic acid and its fluorescence allows quantification of the nucleic acid. To free the nucleic acid, the complex is treated with a polyanion polymer such as dextran sulfate. Heparin, or heparan sulfate, which will "open" up or relax the complex from its condensed state can also be used. This reaction takes typically 10-15 minutes to complete. Picogreen is then added to quantify the exposed nucleic acid. A nucleic acid standard curve is set up with the range between 0.2 μg to 1 μg (*see*, Fig 4.). At each point, a standard amount of dextran sulfate is added. This addition is to offset the quenching effect dextran sulfate has on the fluorescence readings of the picogreen. This quantity is the same amount used to dissociate a PEI/nucleic acid sample. In this way, the nucleic acid associated with the polycatonic polymer can be quantitated (*see*, Fig 2). In Figure 2, the clear bar in Samples 1 and 2 represents the picogreen fluorescence *i.e.*, background.

Figure 3 illustrates the titration of dextran sulfate to determine the minimal amount of dextran sulfate required to release the condensed PEI-nucleic acid complex and completely expose the complexed nucleic acid. This allows accurate quantification of the nucleic acid using a picogreen assay. At least three times more dextran sulfate than PEI is required to completely expose the DNA to picogreen. This represents a charge ratio for PEI/nucleic acid of approximately 5:1. Various charge ratios of PEI/nucleic acid complexes require differing amounts of dextran sulfate. In this manner, the charge ratio can be calculated.

D. Encapsulation of the Condensing Agent-Nucleic Acid Complex

In another embodiment, the present invention relates to a method for encapsulating a condensing agent-nucleic acid complex in a liposome, said method comprising: adding a condensing agent solution into a nucleic acid solution to form a

condensing agent-nucleic acid complex; and adding said condensing agent-nucleic acid complex to a lipid suspension to form an encapsulated condensing agent-nucleic acid complex. In a preferred embodiment, the method comprises:

(a) admixing a condensing agent solution into a nucleic acid solution to form precondensed nucleic acid;

(b) adding the precondensed nucleic acid into a condensing agent solution to form condensing agent-nucleic acid complex;

(c) dialyzing the condensed nucleic acid complex to form concentrated condensing agent-nucleic acid complex;

(d) adding said concentrated condensing agent-nucleic acid complex to a lipid suspension in a detergent; and

(e) removing said detergent from the lipid suspension to form an encapsulated condensing agent-nucleic acid complex in the liposome.

Liposomal encapsulation of the condensing agent-nucleic acid complex offers protection against enzymatic digestion and gives consistently higher gene expression than other transfer methods. To optimize the transfection capability of condensing agent-nucleic acid complexes, the overall charge of the complexes needs to be positive. Unfortunately, with large positive charge ratios, the complexes are toxic and do not last very long in circulation. Thus, it has now been discovered that encapsulation of the condensing agent-nucleic acid complexes in liposomes can reduce the toxicity level of the complexes down to acceptable values.

A variety of lipids can be used in the liposomes of the present invention. Preferably, non-cationic lipids are used. Such lipids include, but are not limited to, phosphatidylethanolamines, phosphatidylserines, ceramides and mixtures thereof. These include, for example, dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylserine (DOPS) and mixtures thereof. Other examples of preferred anionic lipids suitable for use in the present invention include, but are not limited to, cardiolipin, diacylphosphatidic acid, N-succinyl-phosphatydylethanolamine (N-succinyl-PE), phosphatidic acid, phosphatidylinositol, phosphatidylglycerol, phosphatidyl ethylene glycol and mixtures thereof.

Phosphatidylethanolamines and phosphatidylserines containing saturated or unsaturated fatty acids with carbon chain lengths in the range of about C_6 to C_{24} are preferred. Fatty acids with carbon chain lengths in the range of about C_{14} to C_{20} are especially preferred. Phosphatidylethanolamines with mono- or di-unsaturated fatty acids

and mixtures of saturated and unsaturated fatty acids can also be used. Suitable phosphatidylethanolamines include, but are not limited to, dimyristoylphosphatidylethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE) and distearoylphosphatidyl-ethanolamine (DSPE). Dioleoylphosphatidylethanolamine is a preferred phosphatidylethanolamine. The preferred phosphatidylserine is dioleoylphosphatidylserine.

Ceramides suitable for use in accordance with the present invention are commercially available from a number of sources. In addition, ceramides can be isolated, for example, from egg or brain using well-known isolation techniques or, alternatively, they can be synthesized using the methods and techniques disclosed in U.S. Patent No. 5,820,873, issued October 13, 1998, the teachings of which are incorporated herein by reference. Using the synthetic routes set forth in the foregoing application, ceramides having saturated or unsaturated fatty acids with carbon chain lengths in the range of C_6 to C_{24} can be prepared. Preferred ceramides have acyl chain lengths of about C_{14} to about C_{20}.

Phosphatidylethanolamines having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be conjugated to polyethyleneglycol to form the bilayer stabilizing component. Such phosphatidylethanolamines are commercially available, or can be isolated or synthesized using conventional techniques known to those of skill in the art.

Ceramides having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be coupled to polyethyleneglycol to form the bilayer stabilizing component. It will be apparent to those of skill in the art that in contrast to the phosphatidylethanolamines, ceramides have only one acyl group which can be readily varied in terms of its chain length and degree of saturation.

In addition, the liposome contains a bilayer stabilizing component. Examples of suitable bilayer stabilizing components include, but are not limited to, lipid, lipid-derivatives, detergents, polyethylene glycol (PEG), proteins, peptides, polyamide oilgomers, (*e.g.*, ATTA) and pH sensitive oilgomer (*e.g.*, PEAA). (*see*, U.S. Application Serial Nos. 08/996,783 filed December 23, 1997, 60/073,852 filed February 2, 1998, and 60/083,294, filed April 28, 1998, the teachings of which are incorporated herein by reference). In a presently preferred embodiment, the bilayer stabilizing component is polyethyleneglycol conjugated to, *i.e.*, coupled to, a phosphatidylethanolamine or

phosphatidylserine. In an equally preferred embodiment, the bilayer stabilizing component is polyethyleneglycol conjugated to a ceramide. Polyethyleneglycol can be conjugated to a phosphatidylethanolamine, phosphatidylserine or, alternatively, to a ceramide using standard coupling reactions known to and used by those of skill in the art. In addition, preformed polyethylene-glycol phosphatidylethanolamine conjugates are commercially available from Avanti Polar Lipids (Alabaster, Alabama).

Polyethyleneglycols of varying molecular weights can be used to form the bilayer stabilizing components of the present invention. Polyethyleneglycols of varying molecular weights are commercially available from a number of different sources or, alternatively, they can be synthesized using standard polymerization techniques well-known to those of skill in the art. In a presently preferred embodiment, the polyethylene glycol has a molecular weight ranging from about 550 to about 8500 daltons, and even more preferably from about 2000 to about 5000 daltons. Generally, it has been found that increasing the molecular weight of the polyethyleneglycol reduces the concentration of the bilayer stabilizing component required to achieve stabilization.

In addition to the foregoing, polyamide oilgomers, (*e.g.*, ATTA), pH sensitive oilgomers, (*e.g.*, PEAA), detergents, proteins and peptides can be used as bilayer stabilizing components. Detergents which can be used as bilayer stabilizing components include, but are not limited to, Triton X-100, deoxycholate, octylglucoside and lyso-phosphatidylcholine. Proteins which can be used as bilayer stabilizing components include, but are not limited to, glycophorin and cytochrome oxidase. Cleavage of the protein, by endogenous proteases, resulting in the loss of the bulky domain external to the bilayer would be expected to reduce the bilayer stabilizing ability of the protein. In addition, peptides which can be used as bilayer stabilizing components include, for example, the pentadecapeptide, alanine-(aminobutyric acid-alanine)$_{14}$. This peptide can be coupled, for example, to polyethyleneglycol, which would promote its transfer out of the bilayer. Alternatively, peptides such as cardiotoxin and melittin, both of which are known to induce non-lamellar phases in bilayers, can be coupled to PEG and might thereby be converted to bilayer stabilizers.

Typically, the bilayer stabilizing component is present at a concentration ranging from about 0.05 mole percent to about 50 mole percent. In a presently preferred embodiment, the bilayer stabilizing component is present at a concentration ranging from 0.05 mole percent to about 25 mole percent. In an even more preferred embodiment, the bilayer stabilizing component is present at a concentration ranging from 5 mole percent to

about 15 mole percent. One of ordinary skill in the art will appreciate that the concentration of the bilayer stabilizing component can be varied depending on the bilayer stabilizing component employed.

One method of encapsulating the complexes of this invention is by using detergent dialysis. Typically, the encapsulation of the complexes is accomplished by dissolving the lipids in a solvent and then drying the solution under a stream of nitrogen. Preferably the lipids are non-cationic lipids. More preferably, the lipids are DOPE, DOPS, PEG-ceramide and mixtures thereof. The ratio of lipid to nucleic acid is about 5:1 wt/wt to about 100:1 wt/wt, preferably about 10:1 wt/wt to about 50:1 wt/wt. Final total lipid concentration desired is about 10 mg/mL. A thin lipid film is achieved by including a mixing step, such as vortexing, in the drying procedure. Any remaining solvent is removed by freeze-drying.

The lipid film is then dissolved in a detergent, or alternatively, ethanol. The condensing agent-nucleic acid complex is then added, such as a PEI/nucleic acid complex having a nucleic acid concentration of about 50 μg/mL to about 1000 μg/mL. A nucleic acid concentration of about 400 μg/mL is preferred. The resulting mixture is then vortexed until it becomes clear and then dialyzed. This procedure results in a liposome encapsulating the condensing agent-nucleic acid complex. The method can be scaled up proportionately for use in larger preparations.

The detergents that are useful for encapsulating the condensing agent-nucleic acid complexes in the present invention are typically one or more neutral detergents or combinations of detergents and organic solvents. The detergents are preferably, N,N'-((octanoylimino)-bis-(trimethylene))-bis-(D-gluconamide) (BIGCHAP); BRIJ 35; Deoxy-BIGCHAP; dodecylpoly(ethylene glycol) ether; Tween 20; Tween 40; Tween 60; Tween 80; Tween 85; Triton X-405; hexyl-, heptyl-, octyl- and nonyl-β-D-glucopyranoside; with octyl β-D-glucopyranoside being the most preferred.

The organic solvents that are useful in combination with a detergent include, but are not limited to, chloroform, dichloromethane, diethylether, cyclohexane, cyclopentane, benzene, toluene, acetone, benzyl alcohol, methanol, or other aliphatic alcohols such as propanol, *iso*-propanol, butanol, *tert*-butanol, *iso*-butanol, pentanol and hexanol. The selection of an organic solvent will typically involve consideration of solvent polarity and the ease with which the solvent can be removed at the later stages of encapsulation. Accordingly, the preferred organic solvents used in conjunction with the

detergent are ethanol, dichloromethane, chloroform, methanol and diethyl ether with chloroform and methanol being the most preferred.

The solution of non-cationic lipids, bilayer stabilizing component and detergent is an aqueous solution. Contacting the condensing agent-nucleic acid complex with the solution of non-cationic lipids and detergent is typically accomplished by mixing together a first solution of nucleic acids and a second solution of the lipids and detergent. One of skill in the art will understand that this mixing can take place by any number of methods, for example, by mechanical means such as by using vortex mixers. Preferably, the nucleic acid solution is also a detergent solution.

In an alternative embodiment, a dehydration-rehydration method can be used to encapsulate the condensing agent-nucleic acid complex. In this method, a lipid mixture in a solvent is dried down and then rehydrated in a buffer containing the condensing agent-nucleic acid complex. Extrusion of the liposome follows the rehydration step. The dehydration-rehydration method generates lower encapsulation efficiency than the detergent dialysis technique described above.

In yet another embodiment, the reverse phase evaporation method can be employed to encapsulate the complex. In this method, the lipids are first dissolved in a solvent system or mixed solvent system. The condensing agent-nucleic acid complex is dissolved in water and then added to the lipid mixture. The solvent system is added until a single phase is observed. After excess solvent is removed by evaporation, the solution is extruded to yield encapsulated liposomes.

In still yet another embodiment, the ethanol injection method can be used for encapsulation of the complex. In this method, the lipids are dissolved in ethanol, or another suitable solvent, and dripped into a tube containing the condensing agent-nucleic acid complexes in water. The liposomes are formed immediately and the ethanol is dialyzed away to yield encapsulated liposomes.

The size of the liposomes of the present invention are about 20 nm to about 200 nm in diameter. More preferably, the liposomes of the present invention are about 50 nm to about 150 nm in diameter. In an especially preferred embodiment, the liposomes of the present invention are about 70 nm to about 80 nm in diameter.

The size distribution of the condensing agent-nucleic acid complexes and liposomes can be measured by quasielastic light scattering using a Nicomp Submicron Particle Sizer (Model 370) in the solid particle mode and vesicle mode, respectively.

To measure the encapsulation efficiency of the liposomes using the methods above, picogreen and dextran sulfate are used. The amount of unencapsulated complexes M_{uncap} (determined from fluorescence of picogreen) can then be quantified via the combination of dextran sulfate and picogreen. By adding Triton X-100, which completely breaks apart the liposomes, it is also possible to quantify the total DNA present, M_{tot}. The extent of encapsulation is then calculated via the formula:

$$\% \text{ Encapsulation} = (1 - M_{uncap}/ M_{tot.}) \times 100$$

Encapsulation efficiency is best described with reference to Figure 6. As shown in Figure 6, when picogreen is added to an unencapsulated complex, fluorescence is in the background. When dextran sulfate is added to an unencapsulated complex, there is an increase in fluorescence to 3.0. When Triton X-100 is added to break up the liposomes, a further jump in fluorescence occurs (*see*, Sample 1). The ratio of these peaks gives the extent of encapsulation. To remove any unencapsulated complex, a cation exchange column can be used.

In using methods of the present invention, it is possible to encapsulate about 30% to about 70% of the condensing agent-nucleic acid complex . Preferably, percent encapsulation is about 40% to about 70% and most preferably, percent encapsulation is about 50% to about 70%.

With reference to Figure 7, the titration of DOPS to optimize encapsulation efficiency is illustrated. In certain embodiments, a concentration of approximately 8-9 mol% of DOPS in the liposome gives the best entrapment of the complexes, in this case PEI/DNA. Shifting this point by more than 2% in concentration of DOPS drops the encapsulation efficiency dramatically.

E. Administration of Liposome-Entrapped Complexes

Following formation of the liposomal entrapped condensing agent-nucleic acid complexes, the liposomes can be used to transfect cells by contacting the cells to be transfected with the liposomes. The liposome-entrapped complexes can be adsorbed to almost any cell type. Once adsorbed, the liposomes can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, destabilized or fuse with the cells. Transfer or incorporation of the nucleic acid portion of the liposome can take place via any one of these pathways. In particular, when fusion takes place, the lipid bilayer membrane is integrated into the cell membrane and the contents of the bilayer combine with the intracellular fluid. Fusion of the liposome with the plasma membrane takes

place when the bilayer stabilizing component transfers out of the liposome and the bilayer stability is lost or decreased. Without being bound to any theory, polycationic mediated gene transfer is thought to involve DNA aggregation and binding of the resulting complex to anionic residues on the plasma membranes. To be efficient, the complex should bear a net positive charge.

Contact between the cells and the liposomal entrapped condensing agent-nucleic acid complexes, when carried out *in vitro*, will take place in a biologically compatible medium.

Treatment of the cells with the liposome-entrapped complex will generally be carried out at physiological temperatures (about 37°C) for periods of time ranging from about 1 to 48 hours, preferably from about 2 to 4 hours. For *in vitro* applications, the delivery of nucleic acids can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells will be animal cells, more preferably mammalian cells, and most preferably human cells.

With reference to Figure 9, Cos-7 cells were transfected with 1 μg of encapsulated pINEX/L018 plasmid DNA complexed with PEI at the 1:4 w/w ratio. In a dose response and time course analyses, Figure 9 shows that transfection activity increased as DNA dose increased. The highest transfection activity was observed at 5 μg of DNA. Minimal transfection was seen at the 24 hour time point. The transfection activity continued to increase up to the 72 hour time point.

F. Pharmaceutical Preparations

The liposome-entrapped condensing agent-nucleic acid complexes of the present invention can be administered alone or in mixture with a physiologically acceptable carrier. Such carriers include, but are not limited to, physiological saline or phosphate buffer selected in accordance with the route of administration and standard pharmaceutical practice.

Pharmaceutical compositions comprising the liposome-entrapped condensing agent-nucleic acid complexes are prepared according to standard techniques and further comprise a pharmaceutically acceptable carrier. Generally, normal saline will be employed as the pharmaceutically acceptable carrier. Other suitable carriers include, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* In

compositions comprising saline or other salt containing carriers, the carrier is preferably added following liposome formation. Thus, after the liposome-entrapped complexes are formed, the liposome can be diluted into pharmaceutically acceptable carriers, such as normal saline. These compositions may be sterilized by conventional sterilization techniques. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, *etc.*

The concentration of the liposome-entrapped complexes in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.05%, usually at or at least about 2-5% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. For diagnosis, the amount of liposome-entrapped complex administered will depend upon the particular label used, the disease state being diagnosed and the judgement of the clinician but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight.

In another example of their use, the liposomal entrapped condensing agent-nucleic acid complexes can be incorporated into a broad range of topical dosage forms including, but not limited to, gels, oils, emulsions and the like. For instance, the suspension containing the liposomal entrapped condensing agent-nucleic acid complexes can be formulated and administered as topical creams, pastes, ointments, gels, lotions and the like.

The present invention also provides liposome-entrapped condensing agent-nucleic acid complexes in kit form. The kit will typically be comprised of a container which is compartmentalized for holding the various elements of the kit. The kit will contain the liposomes of the present invention, with instructions for administration. In still other embodiments, the liposomal entrapped condensing agent-nucleic acid complexes will have a targeting moiety attached to the liposome. Methods of attaching

targeting moieties (*e.g.*, antibodies, proteins) to lipids (such as those used in the present invention) are known to those of skill in the art.

Dosage for liposome-entrapped condensing agent-nucleic acid complexes will depend on the ratio of nucleic acid to lipid and the administrating physician's opinion based on age, weight, and condition of the patient.

The invention will be described in greater detail by way of specific examples. The following examples are offered for illustration purposes only and are not intended to limit the invention in any manner.

G. Examples

I. Materials

The reporter gene plasmid used in all experiments was pINEX/L018 (5650 bp), which encodes firefly luciferase under the control of the human cytomegalovirus immediate-early enhancer/promoter. Plasmid DNA was prepared from *E. coli* DH5alpha by alkaline lysis followed by double banding on cesium chloride gradients. (*See*, Thierry A.R., *J. Liposome Research* 7:143-159 (1997), incorporated herein by reference.) PEI 25 kDa was purchased from Aldrich Chemical Co. (Milwaukee, WI). Ethidium bromide picogreen was obtained from Sigma Chemical Co. (St. Louis, MO). Purified firefly luciferase was purchased from Boehringer Mannheim (Germany). All lipids, with exception of the PEG-ceramide (made in-house), are obtained from Avanti Lipids. Fetal bovine serum was purchased from Intergen (New York, USA). All culture media were purchased from Stemcell Technology (Vancouver, BC). Other reagents in this study were from Sigma Chemical Co., and used without further purification.

II. Methods

a. Method for Preparing PEI/plasmid DNA complexes.

1.) PEI solution (1.6 mg PEI/mL water): 0.1 volume of the PEI solution was added dropwise, using a syringe fitted with a needle size 26G3/8 gauge, into a plasmid DNA solution (1 mg plasmid DNA/1.25 mL water) while vortexing. 2.) The precondensed PEI/DNA made in step 1, was then added dropwise, using a syringe fitted with a needle size 26G3/8 gauge, into PEI solution (390 g of stock PEI diluted in 500 mL water) while vortexing to form PEI/plasmid DNA complexes. The resulting surface charge of the PEI/DNA complex is a net positive charge. 3.) The PEI/plasmid DNA complex was then transferred into a dialysis bag (6000 - 8000 molecular weight cut-off,

Spectra-Por, Spectrum) and covered with a drying agent (polyethylene glycol, 10,000 molecular weight). 4.) The concentrated PEI/plasmid DNA complexes was dialyzed overnight against HBS (150 mM NaCl, 5 mM - 25 mM HEPES), pH 7.45 to adjust the NaCl concentration to 150 mM.

ii. Method for the Quantification of Plasmid DNA.

The PEI/plasmid DNA complexes were treated with a polyanion polymer such as dextran sulfate in water (other polymers such as heparin or heparan sulfate can also be used) where for every 4 μg PEI used, 40 μg of dextran sulfate is used. This reaction takes typically between 10 to 15 minutes to complete. 4 μl picogreen is then added to the PEI/plasmid DNA complex. A DNA standard curve is set up with a range running between 0.2 μg to 1.0 μg, where at each point, a standardized amount of dextran sulfate is added. This is to offset the quenching effect of the dextran sulfate on the fluorescence readings of the picogreen. This amount must also be the same as the amount used to dissociate the PEI/plasmid DNA sample.

iii. Protocol for Encapsulating the PEI/plasmid DNA Complexes in Lipids.

The lipids, DOPE (82 mol %), DOPS (8 mol %), and Peg-Ceramide(C20) (10 mol %), dissolved in chloroform, were first dried under a stream of nitrogen. Final total lipid concentration desired is 10 mg/mL. A thin lipid film is achieved by including vortexing in the drying procedure. Any remaining solvent was removed by further freeze-drying overnight. The dried-down lipid film is then removed from the lyophilizer and 200 μl of OGP (200 mM) was added. Hard vortexing, followed by warming at 65°C for 5 minutes intermittently, helps to dissolve the lipids in the detergent. When no apparent undissolved lipid can be seen, the PEI/DNA complexes in the DNA concentration of 400 μg/mL, is then added to the lipid suspension. The lipid:DNA w/w ratio used is 10mg:400 μg respectively. Anything above the concentration of 500μg/mL DNA was found to cause precipitation or flocculation to occur (final product has big particles which disappeared momentarily upon shaking). The resulting mixture is then vortexed until it becomes clear and then transferred into a Spectra-Por dialysis bag for dialysis. The dialysis buffer is made up of 5 mM Hepes, 150 mM NaCl and titrated with appropriate amounts NaOH to achieve a pH of approximately 7.45. The mixture is then dialyzed for 24 hours with buffer changes at every 4 hours.

iv. Method for Determining Encapsulation Efficiency of the Lipid Formulation.

To measure the encapsulation efficiency of lipid particles, picogreen and dextran sulfate were used. The amount of unencapsulated complexes M_{uncap} (determined from fluorescence of picogreen) could be quantified via the combination of dextran sulfate and picogreen. When Triton X-100 was added, which completely dissociates the lipid particles, the total DNA present, M_{tot} could be determined. The extent of encapsulation is then calculated using the formula:

$$\% \text{ Encapsulation} = (1 - M_{uncap}/ M_{tot}) \times 100$$

v. Protocol for treating the Encapsulated PEI/plasmid DNA Lipid Particles.

The cation gel, about 100 mL (Dowex-50W, Catalog No. 50X8-400, Sigma) is first placed in a volumetric flask (1000 mL capacity) containing approximately 500 mL of 0.5 M HCl, which acts as a proton reservoir. A magnetic stir bar was included in the flask and the mixture was stirred slowly on a stir-plate overnight. The following day, the gel is then loaded into a chromatographic column (GlassEcono-column, Catalog No. 737-1012, BioRad) to a height of about 5 cm. The gel is then washed with 10 volumes of distilled water to normalize the pH of the gel. After the wash, 100 mL of 5 M NaCl is used to wash off any remaining impurities on the gel. Finally, 10 column volumes of 150 mM NaCl HBS was used to equilibrate the column.

v. Method for Transfection of the PEI/plasmid DNA Lipid Particles Using Several Cell Lines *in vitro*.

Lewis Lung, SK-OV-3, LS180, Cos 7, B16 and U87 cells were seeded in 24-well plates (Falcon 3047) at a density of 4×10^4, 2×10^4, 4×10^4, 2×10^4, 4×10^4 and 4×10^4 cells/well in 1 mL of media with 10% fetal bovine serum. B16 and LS180 cells were grown in MEM + Earls Salts, Lewis Lung , Cos 7 and U87 cells were grown in DMEM with high glucose, and SK-OV-3 cells were grown in RPMI 1640 medium. Cells were incubated overnight to 70-80% confluent at the time of transfection. The encapsulated PEI condensed DNA lipid formulations were prepared as described above and were added to the appropriate wells in triplicate. The plates were agitated briefly then incubated at 37°C (5% CO_2) for 24 and 48 hours before assaying for gene expression.

Luciferase gene expression was measured with a luminometer (Dynatech Microlite TM ML3000 Microtiter) using 96-well plates (Catalog No. 011-010-7411,

Dynatech). Cells were rinsed once with PBS buffer, and were then lysed with 150 mL of lysis buffer (0.1% Triton X-100, 250 mM sodium phosphate buffer, pH 8.0) at room temperature for 10 - 15 minutes. Duplicate assays for 10 µl of cell lysate were performed. A standard curve was prepared using purified luciferase protein diluted into mock transfected cell lysate.

vi. Method for Measuring the Total Protein of Each *in vitro* Transfected Sample

Protein assay was performed by using the bicinchoninic acid (BCA) colorimetric method. In this assay, 10 µl of lysate was transferred to the individual wells of 96-well plate (Catalog No. 011 - 010-7411, Dynatech), 200 µl of Micro BCA working reagent was added to each well, mixed and incubated at 37°C for 2 hours. The amount of protein in each well was determined by comparison with BSA protein standard (1-16 µg/well) added to a series of duplicate wells on the same plate. The plate with samples and BSA protein standard was read at 570 nm in a microtiter plate reader (Dynatech MR5000) after allowing the color to develop.

vii Method of Evaluating the Level of Toxicity *in vitro*

Dilute 0.1% crystal violet reagent to 0.05% with 20% ethanol. Centrifuge plates at 1500 rpm for 10 minutes. Rinse plates 2 times with PBS buffer by pouring the buffer into the lid of a pipette tip box and submerging the plates. Change the PBS buffer between plates. Invert plates onto a stack of paper towels and gently pat to dry. Add 50-100 µl of 0.05% crystal violet to each well. Incubate plates at room temperature for 10 minutes. Rinse plates with tap water as described above for the PBS wash. Allow plates to dry on the bench top overnight. Add 100 µl of 100% methanol to each well. Read plates within 5 minutes of methanol addition using the plate reader at 570nm.

Cos-7 cells were seeded onto 96-well plates at a density of 2.5×10^3 in 200 µl of completed medium and incubated 72 hours to 90% confluence. different amount of encapsulated PEI condensed DNA formulations and PEI/DNA complex were added to the appropriate wells in triplicate. Stained the cells with crystal violet after 24 hours incubated.

viii Method for delivering the PEI/plasmid DNA lipid particles in the *in vivo* system

Female C57 mice were injected intraperiotenially (i.p.) with 1 x 10^5 B16 tumor cells. On day 7 of B 16 tumor growth, DNA doses of 75 (g luciferase plasmid/ formulation (encapsulated PEI/DNA lipid particles) were administered in a volume of 500 µl by intraperitoneal injection. Control animals were injected with the same volume of saline. Tumors were collected at different time points, fast frozen in liquid nitrogen and stored at -70°C until analysis. Individual tumors were homogenized using a FastPrep homogenizer (Bio101 inc.) for 5 seconds at speed setting of 5, loaded with a small bead (Catalog No. 6520-401/404, Bio 101), then a second bead and a certain amount of 1 x CCLR reagent (Cell Culture Lysis Reagent Catalog No. E1531, Promega) supplemented with 1 mg/mL BSA (Catalog No. A-2153, Sigma) was added to each tube. The homogenization was performed twice in the FastPrep instrument (FastPrep™ FP120 Instrument, Bio 101) using a speed setting of 5 for 6 seconds. The homogenate was transferred to fresh Eppendorf tubes and large tissue debris was pelleted to the bottom of the tube by brief centrifugation. 20 µl of homogenate and standard luciferase protein diluted with control tissue homogenate were assayed in duplicates. The results were converted to pg of luciferase protein per organ or gram of tumor.

ix. Method for Evaluating Toxicity in the *in vivo* system by Measuring Levels of Aminotransferase (AST/GOT) Activity in Serum.

1.) Add 10 mL of deionized water to a vial. Mix immediately several times by inversion (not shaking). Store reagent up to 16 hours at room temperature or up to 7 days refrigerated. 2.) Turn the UV lamp on at least one half an hour before analysis. Go to kinetics analysis and use the default program within the kinetics setting. Set spectrophotometer to 340 nm and the times as 30, 60, 90, and 120 seconds. Blank with water. 3.) Add 500 µl of reagent to the cuvette (make sure the reagent is at 25°C). 4.) Add 50 µl of the test serum and mix by pipetting up and down. 5.) Read the absorbance after 60 seconds. This is the initial value (inA). 6.) Read the absorbance 30 seconds after the inA reading. This reading is used to verify a linear reaction. 7.) Read the absorbance 60 seconds after the inA reading. This is the final reading (finA). 8.) Calculate the (A per minute by subtracting FinalA from InitialA. If (A per minute is greater than 0.280, dilute 1 part sample with 1 part isotonic saline and reassay. Multiply the results by 2 to compensate for the dilution. 9.) To calculate the

$$AST(U/L) = \frac{(A \text{ per minute} \times TV \times 1000}{6.22 \times LP \times SVTV} = \text{Total volume (0.55 mL)}$$

SV = Sample volume (0.55 mL)

6.22 = Millimolar absorptivity of NADH at 340 nm

LP = Light path (1.0)

1000 = Conversion of units per mL to units per liter

$$= \frac{(A \text{ per minute} \times 0.55 \times 1000}{6.22 \times 1.0 \times 0.05}$$

=(A per minute x 1786 (x 1.37 if values were determined at 25°C)

One unit of activity is defined as the amount of enzyme which produces 1 (mole of NAD per minute under the conditions of the assay procedure.

EXAMPLE 1

This example illustrates the effect of dextran sulfate on PEI/DNA complexes.

Figure 2 (sample 1) illustrates that when picogreen is added to a sample of the liposome formulation containing a PEI/DNA complex, the fluorescence reading is well in the background. It is clear that the DNA is well protected by the condensing agent PEI. When dextran sulfate is added, a significant jump in fluorescence results. The DNA is clearly not in the same condensed form, thus allowing access to picogreen. In sample 2, when Triton X100 is added, there is no significant change in the fluorescence reading *i.e.*, similar to background. This is an indication that Triton does not affect the complexes in any significant way. Upon addition of dextran sulfate, the fluorescence increases.

EXAMPLE 2

This example illustrates the use of dextran sulfate and the amount required for complex dissociation

As is illustrated in Figure 3, when the amount of dextran sulfate added is increased, the fluorescence also increases. This indicates that more and more DNA is made accessible to the picogreen. The increase in fluorescence eventually tapers off at some level and eventually the fluorescence signal is quenched by the excess dextran sulfate. The optimum dextran sulfate to PEI w/w ratio is around 6:1. It is noted that each point is Figure 3 includes an incubation period of approximately 15 minutes.

EXAMPLE 3

This example illustrates the relaxation time of the PEI-DNA complexes.

Figure 5 illustrates the time relaxation profile of PEI/DNA complexes under the effect of dextran sulfate. The graph shows clearly that the relaxation of the complexes is not an instantaneous event. The initial relaxation is rapid, slowing down eventually to final equilibration, as indicated by the picogreen fluorescence signal. These results indicate that for quantification purposes, the required amount of dextran sulfate must be added in advance to the sample and allowed to incubate for at least 15 minutes, to ensure that the relaxation process is complete.

EXAMPLE 4

This example illustrates the encapsulation efficiency of PEI/DNA complexes into liposomes.

As shown in Figure 7, to optimize the encapsulation efficiency, DOPS was titrated and it was found that between 8-9 mol% DOPS gives the best encapsulation at about 55%. This percent encapsulation was determined prior to column loading. Encapsulation efficiencies drop dramatically below this concentration, indicating the high sensitivity of the procedure on the negative surface-charge density. Note that this was obtained in a buffer with 150 mM NaCl concentration. Changing the NaCl concentration will change the amount of DOPS required to optimize encapsulation. For *in vitro* tests, about 8 mol % of DOPS was optimum.

EXAMPLE 5

This example illustrates the efficiency of transfection of the PEI/DNA complexes *in vitro*.

As shown in Figure 9, Cos-7 cells were transfected with 1 μg of encapsulated pINEX/L018 plasmid DNA complexed with PEI at a 1:4 w/w ratio. In this experiment, dose response and time course were analyzed. Figure 9 shows that activity increased as DNA dose increased. The highest transfection activity was observed at 5 μg of DNA. Minimal transfection was seen at the 24 hour time point. The transfection activity continued to increase up to the 72 hour time point.

EXAMPLE 6

This example illustrates the reduction of toxicity of the encapsulated PEI/DNA complexes.

As shown in Figure 10, a toxicity study was conducted of an encapsulated PEI/DNA complex with a dose response having a complex charge-ratio at 5.3. As an illustration, the time-point selected was 48 hours. The control was the cell line with no added components. The graph clearly shows that unencapsulated complexes shows significant toxicity beginning at the 1 μg dose. The encapsulated complexes showed no relative toxicity up to 2 μg DNA.

As shown in Figure 11, the comparison of toxicity between (1) liposomes not treated by a cation exchange column and (2) those which have been so treated is illustrated. The unencapsulated complexes contains 0.75 μg DNA, which is selected to be exactly what is known to be on the outside of the liposome sample. The toxicity of (1) is seen to be comparable to that of the unencapsulated complexes. Sample (2) shows dramatic reduction in toxicity, and can be attributed to the removal of the complexes by a cation exchange column.

Figure 12 illustrates *in vivo* toxicity of encapsulated PEI/DNA complexes. Encapsulated PEI/DNA with a dose of 75 μg DNA is injected into 4 mice and the enzyme (AST) levels were found to be comparable to that of the PBS control. At a dosage of about 4:1 w/w ratio of PEI/DNA, an unencapsulated sample would have been lethal to the mice. The encapsulation efficiency in this liposomal injection is close to 90%.

All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

Although the invention has been described with reference to preferred embodiments and examples thereof, the scope of the present invention is not limited only to those described embodiments. As will be apparent to persons skilled in the art, modifications and adaptations to the above-described invention can be made without departing from the spirit and scope of the invention, which is defined and circumscribed by the appended claims.

WHAT IS CLAIMED IS:

1. A liposome comprising:

(a) a lipid; and

(b) a condensing agent-nucleic acid complex encapsulated in said liposome.

2. A liposome in accordance with claim 1, further comprising:

(c) a bilayer stabilizing component associated with said liposome.

3. A liposome in accordance with claim 2, wherein said bilayer stabilizing component is reversibly associated with said liposome.

4. A liposome in accordance with claim 1, wherein said lipid comprises a non-cationic lipid.

5. A liposome in accordance with claim 4, wherein said non-cationic lipid is a member selected from the group consisting of phosphatidylethanolamines, phosphatidylserines and mixtures thereof.

6. A liposome in accordance with claim 4, wherein said non-cationic lipid is a member selected from the group consisting of cardiolipin, diacylphosphatidic acid, N-succinyl-phosphatydylethanolamine, phosphatidic acid, phosphatidylinositol, phosphatidylglycerol, phosphatidyl ethylene glycol and mixtures thereof.

7. A liposome in accordance with claim 5, wherein said non-cationic lipid is a member selected from the group consisting of dioleoylphosphatidylethanolamine, dioleoylphosphatidylserine and mixtures thereof.

8. A liposome in accordance with claim 1, wherein said condensing agent is a member selected from the group consisting of polyethylenimine, polylysine, polyarginine, polyornithine, histones, protamines, polyamines, spermidine and spermine.

9. A liposome in accordance with claim 8, wherein said condensing agent is polyethylenimine having a molecular weight of about 0.8 kDa to about 800 kDa.

10. A liposome in accordance with claim 9, wherein said polyethylenimine has a molecular weight of about 10 kDa to about 50 kDa.

11. A liposome in accordance with claim 1, wherein said condensing agent-nucleic acid complex is about 30 nm to about 60 nm in diameter.

12. A liposome in accordance with claim 1, wherein said liposome is about 20 nm to about 200 nm in diameter.

13. A liposome in accordance with claim 12, wherein said liposome is about 50 nm to about 150 nm in diameter.

14. A liposome in accordance with claim 12, wherein said liposome is about 70 nm to about 80 nm in diameter.

15. A liposome in accordance with claim 2, wherein said bilayer stabilizing component is a member selected from the group consisting of a lipid, a lipid-derivative, a detergent, a polyethylene glycol, a protein, a peptide, a polyamide oligomer, a pH sensitive polymer and a PEG-lipid.

16. A liposome in accordance with claim 15, wherein said bilayer stabilizing component is a PEG-lipid.

17. A liposome in accordance with claim 16, wherein said lipid of said PEG-lipid stabilizing component is a member selected from the group consisting of ceramides, phosphatidylethanolamines and phosphatidylserines.

18. A liposome in accordance with claim 17, wherein said PEG-lipid is a PEG-ceramide.

19. A liposome in accordance with claim 18, wherein said PEG-ceramide has an alkyl chain length of about C_6 to about C_{24}.

20. A liposome in accordance with claim 19, wherein said PEG-ceramide has an alkyl chain length of about C_{14} to about C_{20}.

21. A liposome in accordance with claim 16, wherein said PEG is a polyethylene glycol with an average molecular weight of about 550 to about 8500 daltons.

22. A liposome in accordance with claim 21, wherein said PEG has an average molecular weight of about 2000 to about 5000 daltons.

23. A liposome in accordance with claim 9, wherein said polyethylenimine:nucleic acid ratio in said condensing agent-nucleic acid complex is about 10:1 wt/wt to about 1.5:1 wt/wt.

24. A liposome in accordance with claim 23, wherein said polyethylenimine:nucleic acid ratio in said condensing agent-nucleic acid complex is about 6:1 wt/wt to about 1.5:1 wt/wt.

25. A liposome in accordance with claim 23, wherein said polyethylenimine:nucleic acid ratio in said condensing agent-nucleic acid complex is about 4:1 wt/wt.

26. A liposome in accordance with claim 1, wherein said lipid:nucleic acid ratio in said liposome is about 5:1 wt/wt to about 100:1 wt/wt.

27. A liposome in accordance with claim 26, wherein said lipid:nucleic acid weight ratio in said liposome is about 10:1 wt/wt to about 50:1 wt/wt.

28. A liposome in accordance with claim 16, wherein said PEG-lipid comprises about 5 to about 15 mol% of the composition of said liposome.

29. A liposome in accordance with claim 18, wherein said PEG-ceramide comprises about 5 to about 15 mol% of the composition of said liposome.

30. A liposome in accordance with claim 1, wherein said encapsulated condensing agent-nucleic acid complex represents greater than about 30% encapsulation efficiency as determined using picogreen and dextran sulfate.

31. A liposome in accordance with claim 1, wherein said encapsulated condensing agent-nucleic acid complex represents greater than about 40% encapsulation efficiency as determined using picogreen and dextran sulfate.

32. A method of transfecting a cell with a nucleic acid, said method comprising contacting said cell with a liposome comprising:

(a) a lipid; and

(b) a condensing agent-nucleic acid complex encapsulated in said liposome.

33. A method of transfecting a cell with a nucleic acid in accordance with claim 32, wherein said liposome further comprises:

(c) a bilayer stabilizing component associated with said liposome.

34. A method of transfecting a cell with a nucleic acid in accordance with claim 33, wherein said bilayer stabilizing component is reversibly associated with said liposome.

35. A method of transfecting a cell with a nucleic acid in accordance with claim 32, wherein said lipid comprises a non-cationic lipid.

36. A method of transfecting a cell with a nucleic acid in accordance with claim 35, wherein said non-cationic lipid is a member selected from the group consisting of phosphatidylethanolamines, phosphatidylserines and mixtures thereof.

37. A method of transfecting a cell with a nucleic acid in accordance with claim 35, wherein said non-cationic lipid is a member selected from the group consisting cardiolipin, diacylphosphatidic acid, N-succinyl-phosphatydylethanolamine, phosphatidic acid, phosphatidylinositol, phosphatidylglycerol, phosphatidyl ethylene glycol and mixtures thereof.

38. A method of transfecting a cell with a nucleic acid in accordance with claim 36, wherein said non-cationic lipid is a member selected from the group consisting of dioleoylphosphatidylethanolamine, dioleoylphosphatidylserine and mixtures thereof.

39. A method of transfecting a cell with a nucleic acid in accordance with claim 32, wherein said condensing agent is a member selected from the group consisting of polyethylenimine, polylysine, polyarginine, polyornithine, histones, protamines, polyamines, spermidine and spermine.

40. A method of transfecting a cell with a nucleic acid in accordance with claim 39, wherein said condensing agent is polyethylenimine having a molecular weight of about 10 kDa to about 50 kDa.

41. A method of transfecting a cell with a nucleic acid in accordance with claim 32, wherein said condensing agent-nucleic acid complex is about 30 nm to about 60 nm in diameter.

42. A method of transfecting a cell with a nucleic acid in accordance with claim 32, wherein said liposome is about 70 nm to about 80 nm in diameter.

43. A method of transfecting a cell with a nucleic acid in accordance with claim 33, wherein said bilayer stabilizing component is a member selected from the group consisting of a lipid, a lipid-derivative, a detergent, a polyethylene glycol, a protein, a peptide, a polyamide oligomer, a pH sensitive polymer and a PEG-lipid.

44. A method of transfecting a cell with a nucleic acid in accordance with claim 43, wherein said bilayer stabilizing agent is a PEG-lipid.

45. A method of transfecting a cell with a nucleic acid in accordance with claim 44, wherein said lipid of said PEG-lipid stabilizing agent is a member selected from the group consisting of ceramides, phosphatidylethanolamines and phosphatidylserines.

46. A method of transfecting a nucleic acid into a cell in accordance with claim 45, wherein said bilayer stabilizing agent is a PEG-ceramide.

47. A method of transfecting a nucleic acid into a cell in accordance with claim 46, wherein said PEG-ceramide has an alkyl chain length of about C_6 to about C_{24}.

48. A method of transfecting a nucleic acid into a cell in accordance with claim 47, wherein said PEG-ceramide has an alkyl chain length of about C_{14} to about C_{20}.

49. A method of transfecting a nucleic acid into a cell in accordance with claim 44, wherein said PEG has an average molecular weight of about 550 to about 8500 daltons.

50. A method for transfecting a nucleic acid into a cell in accordance with claim 40, wherein said polyethylenimine:nucleic acid ratio in said polyethylenimine-nucleic acid complex is about 10:1 wt/wt to about 1.5:1 wt/wt.

51. A method of transfecting a nucleic acid into a cell in accordance with claim 50, wherein said polyethylenimine:nucleic acid ratio in said polyethylenimine nucleic acid complex is about 4:1 wt/wt.

52. A method for transfecting a nucleic acid into a cell in accordance with claim 32, wherein said lipid:nucleic acid weight ratio in said liposome is about 10:1 to about 50:1.

53. A method for transfecting a nucleic acid into a cell in accordance with claim 44, wherein said PEG-lipid comprises about 5 to about 15 mol% of the composition of said liposome.

54. A method for transfecting a nucleic acid into a cell in accordance with claim 46, wherein said PEG-ceramide comprises about 5 to about 15 mol% of the composition of said liposome.

55. A method for encapsulating a condensing agent-nucleic acid complex in a liposome, said method comprising:

adding a condensing agent solution into a nucleic acid solution to form a condensing agent-nucleic acid complex; and

adding said condensing agent-nucleic acid complex to a lipid suspension to form an encapsulated condensing agent-nucleic acid complex.

56. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim 55, wherein said condensing agent-nucleic acid complex is formed by admixing a first condensing agent to form a precondensed nucleic acid and then adding said precondensed nucleic acid into a second condensing agent solution to form said condensing agent-nucleic acid complex wherein said first and said second condensing agents are the same or different.

57. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim 55, wherein said lipid suspension comprises a non-cationic lipid.

58. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim 55, wherein said condensing agent-nucleic acid complex is about 30 nm to about 60 nm in diameter.

59. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim 55, wherein said lipid suspension comprises a PEG-lipid.

60. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim 58, wherein said PEG-lipid comprises a PEG-ceramide.

61. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim 58, wherein said first condensing agent is polyethylenimine.

62. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim 55, wherein said lipid:nucleic acid ratio in said liposome is about 10:1 wt/wt to about 50:1 wt/wt.

63. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim 58, wherein said PEG-lipid comprises about 5 to about 15 mol% of the composition of said liposome.

64. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with 59, wherein said PEG-ceramide comprises about 5 to about 15 mol% of the composition of said liposome.

65. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim 55, wherein step (c) employs a detergent dialysis.

66. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim 55, wherein step (c) employs an ethanol injection.

Fig. 1

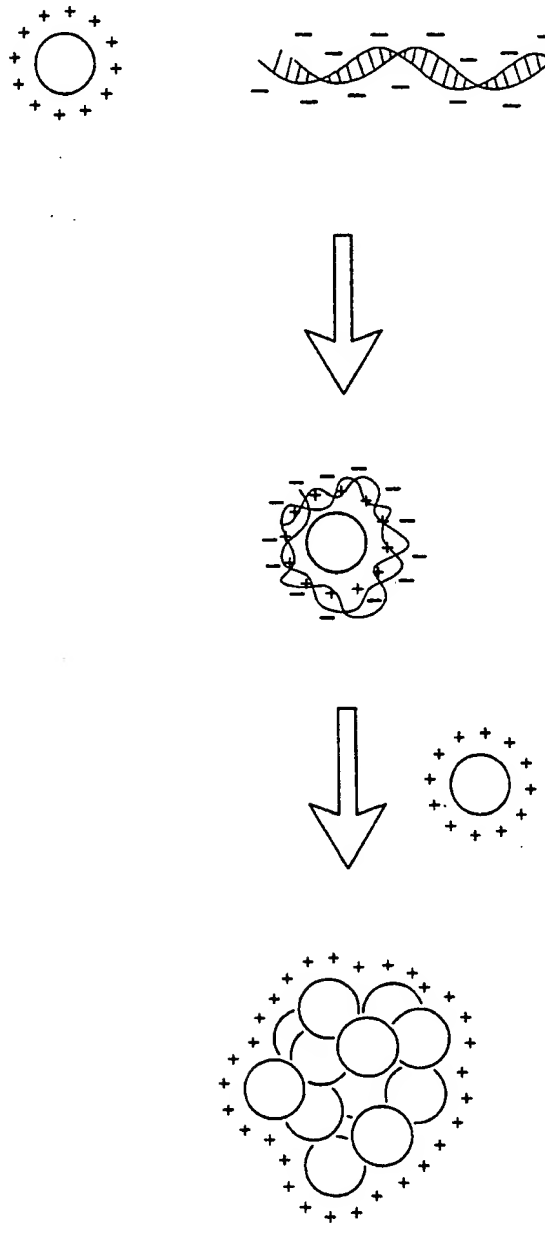


Fig. 2

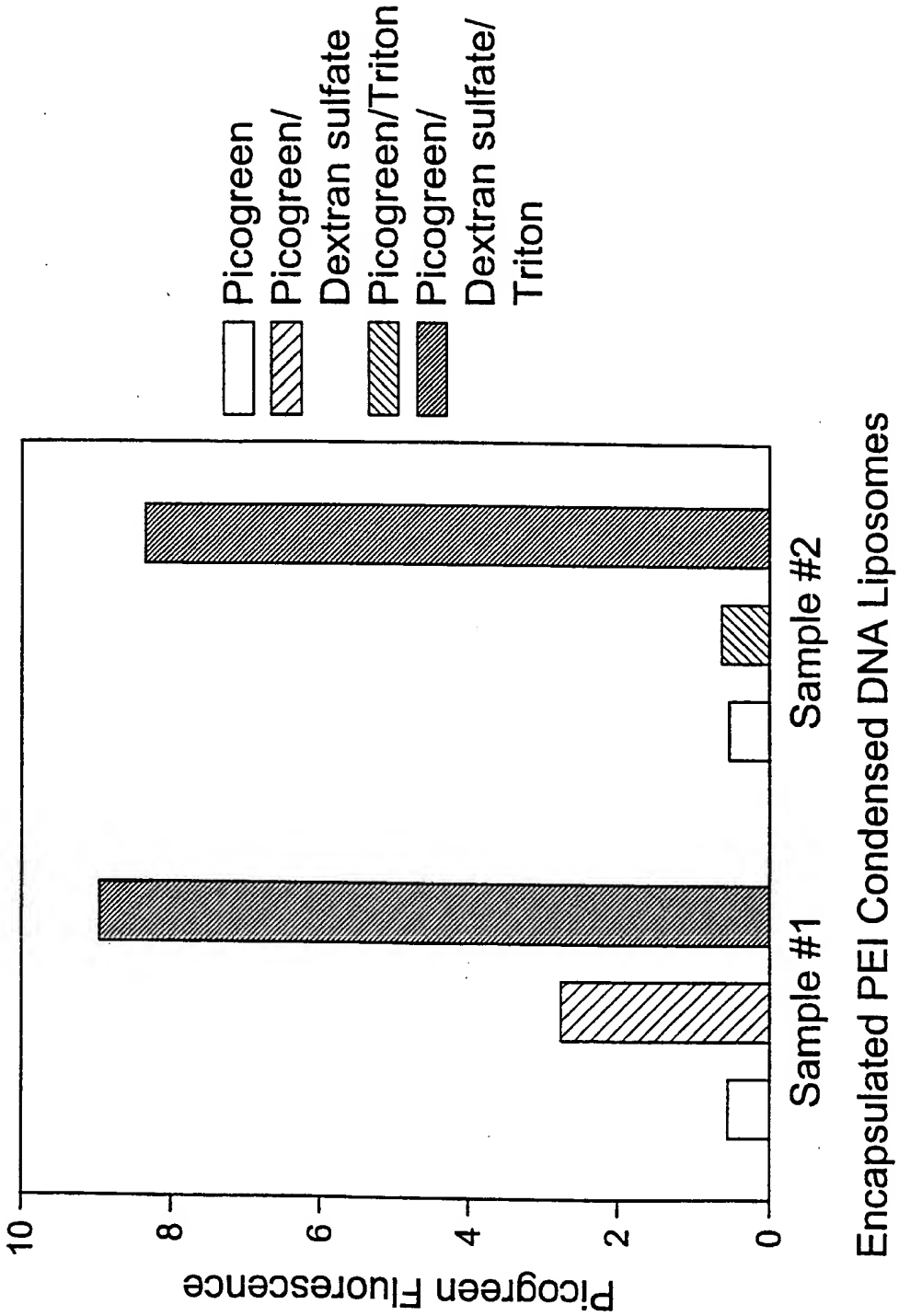

Picogreen
Picogreen/ Dextran sulfate
Picogreen/Triton
Picogreen/ Dextran sulfate/ Triton
Picogreen Fluorescence
0
2
4
6
8
10
Sample #1
Sample #2
Encapsulated PEI Condensed DNA Liposomes

Fig. 3

Titration of Dextran Sulfate to Determine Threshold Amount Required to Unbind PEI Complexes

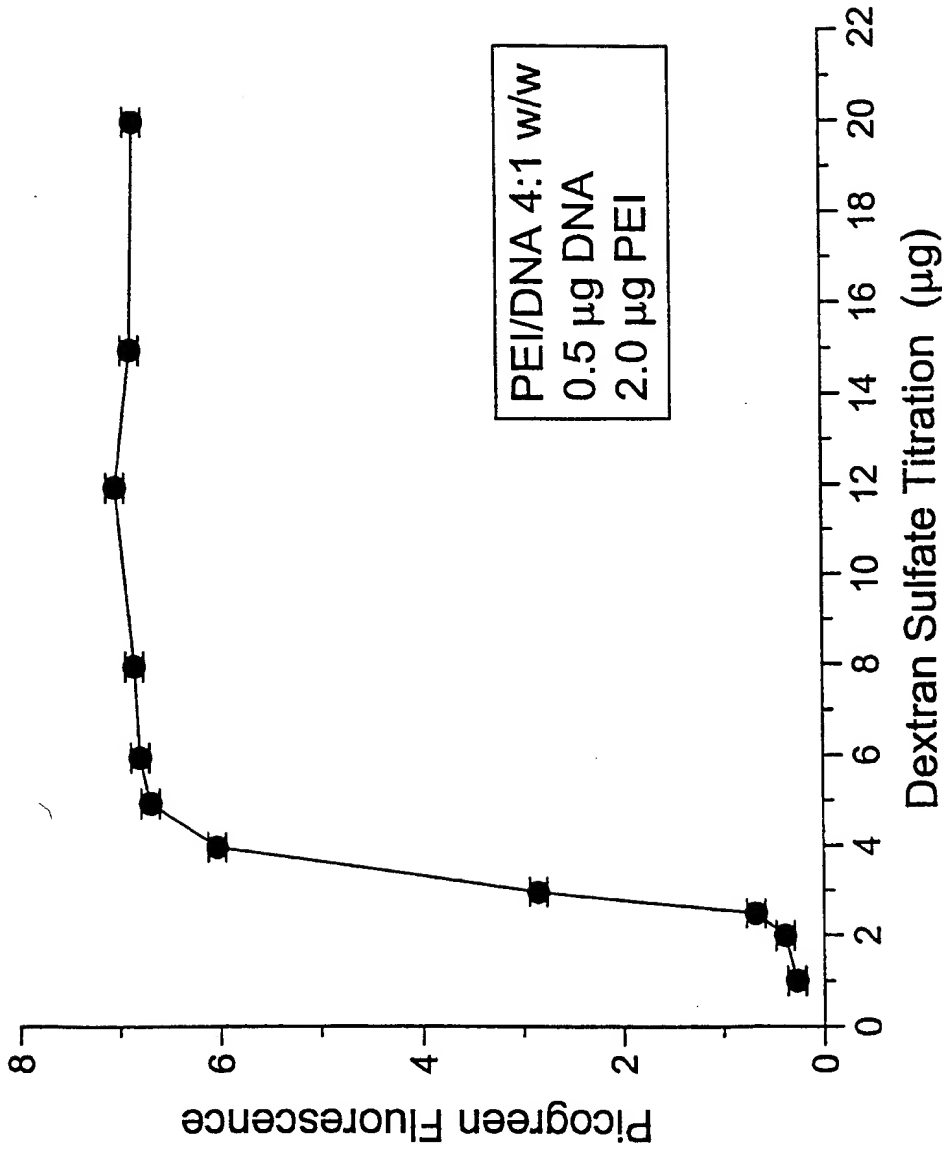


Fig. 4

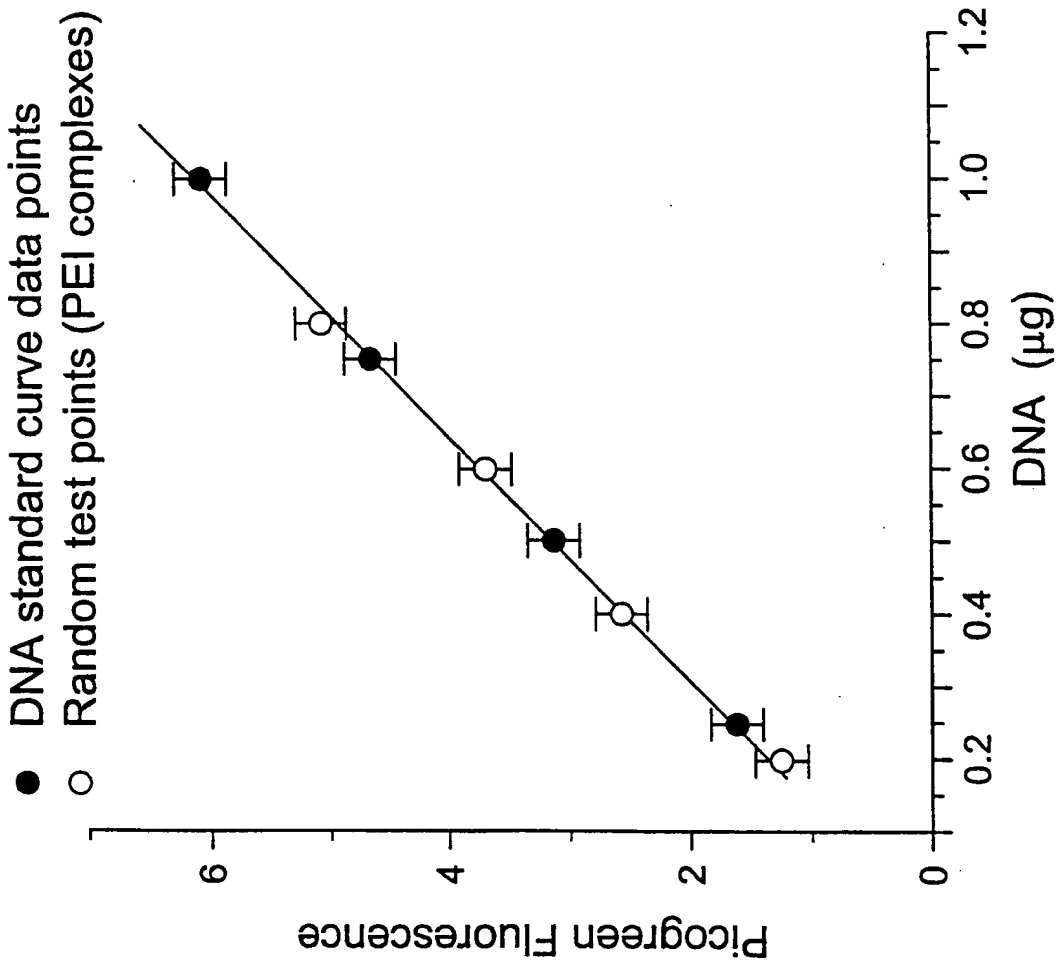
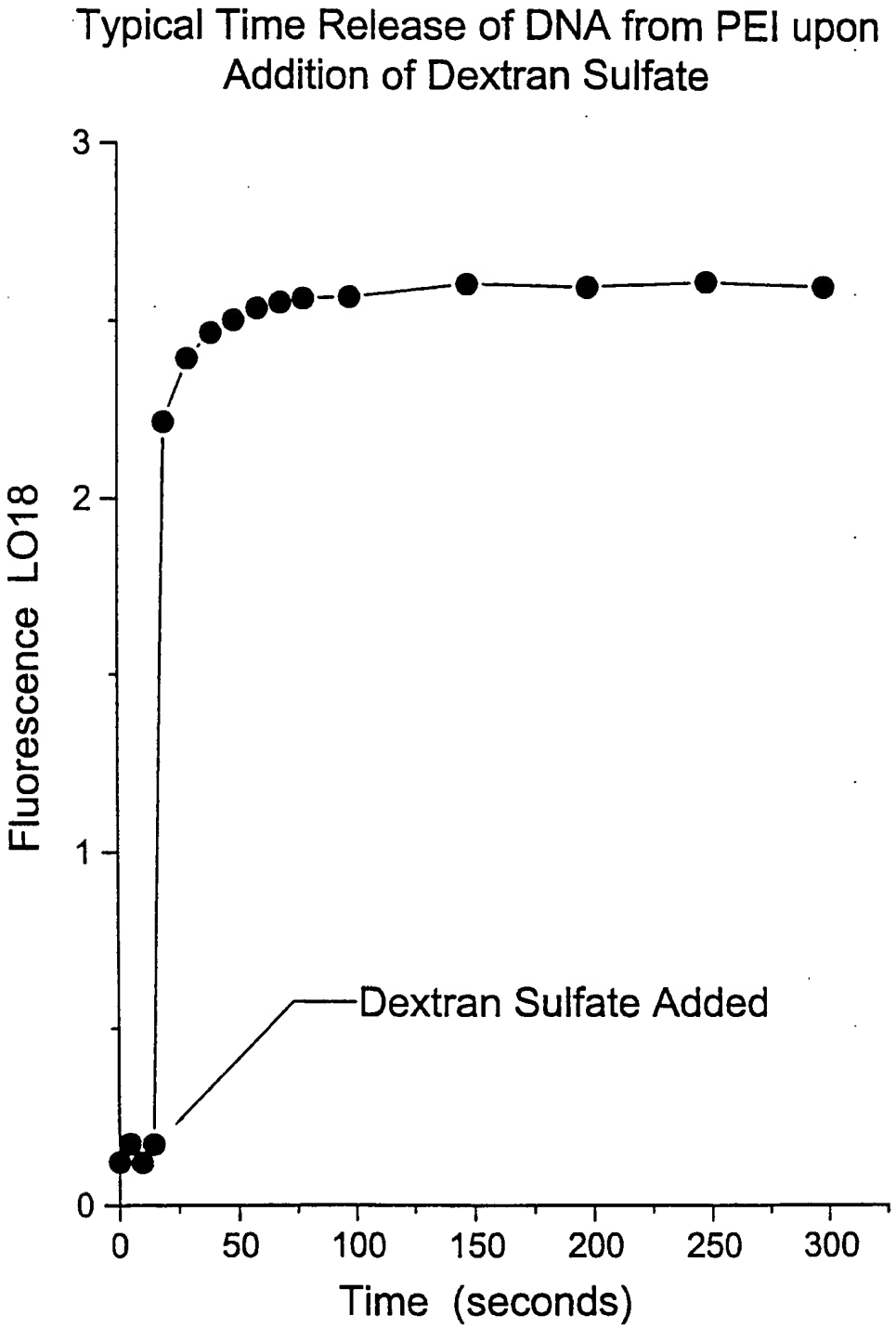

DNA standard curve data points
Random test points (PEI complexes)
Picogreen Fluorescence
0
2
4
6
0.2
0.4
0.6
0.8
1.0
1.2
DNA (µg)

Fig. 5


Typical Time Release of DNA from PEI upon Addition of Dextran Sulfate
Fluorescence LO18
3
2
1
0
0
50
100
150
200
250
300
Time (seconds)
Dextran Sulfate Added

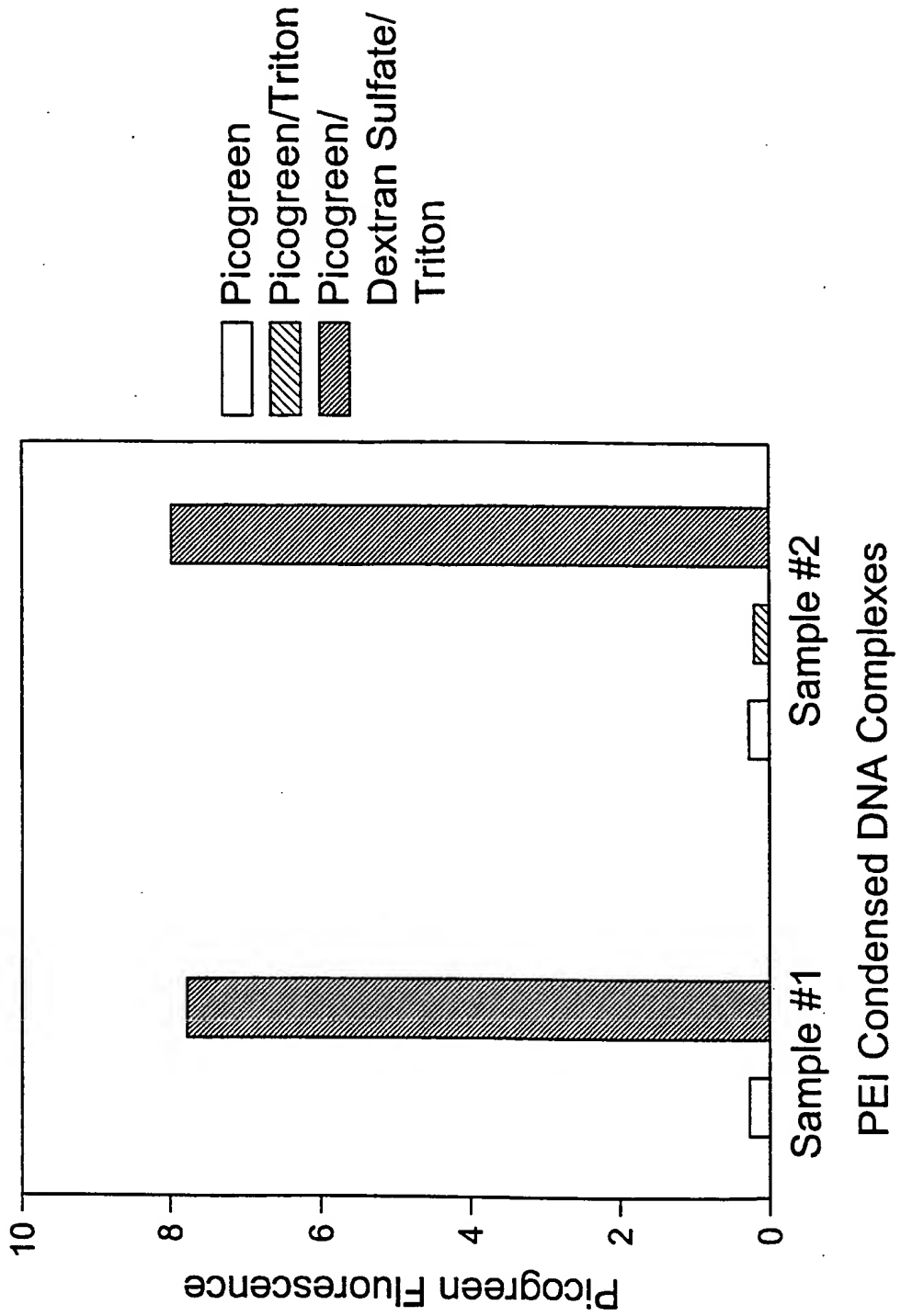

Picogreen
Picogreen/Triton
Picogreen/
Dextran Sulfate/
Triton
Picogreen Fluorescence
0
2
4
6
8
10
Sample #1
Sample #2
PEI Condensed DNA Complexes

Fig. 6

Fig. 7

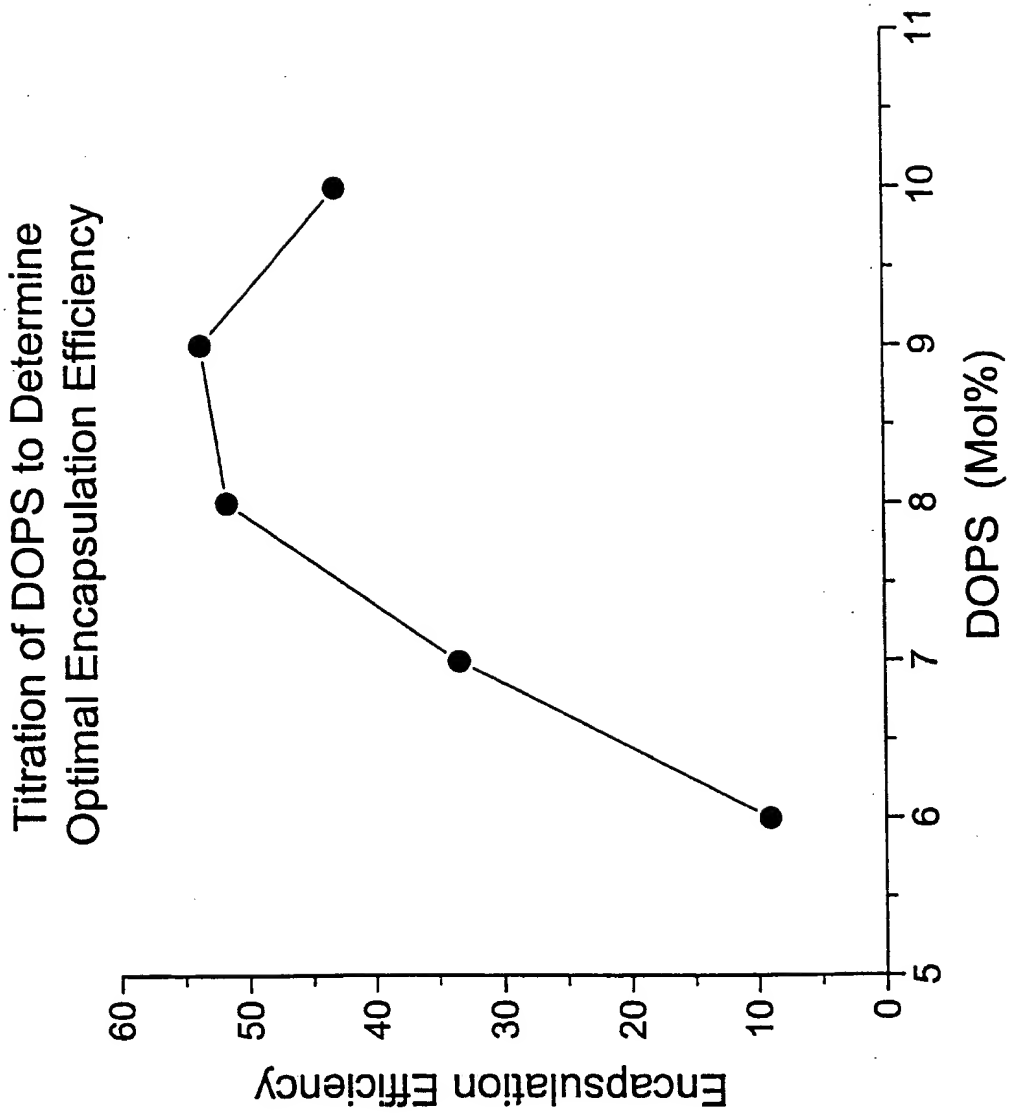

Titration of DOPS to Determine
Optimal Encapsulation Efficiency
Encapsulation Efficiency
0
10
20
30
40
50
60
5
6
7
8
9
10
11
DOPS (Mol%)

Fig. 8

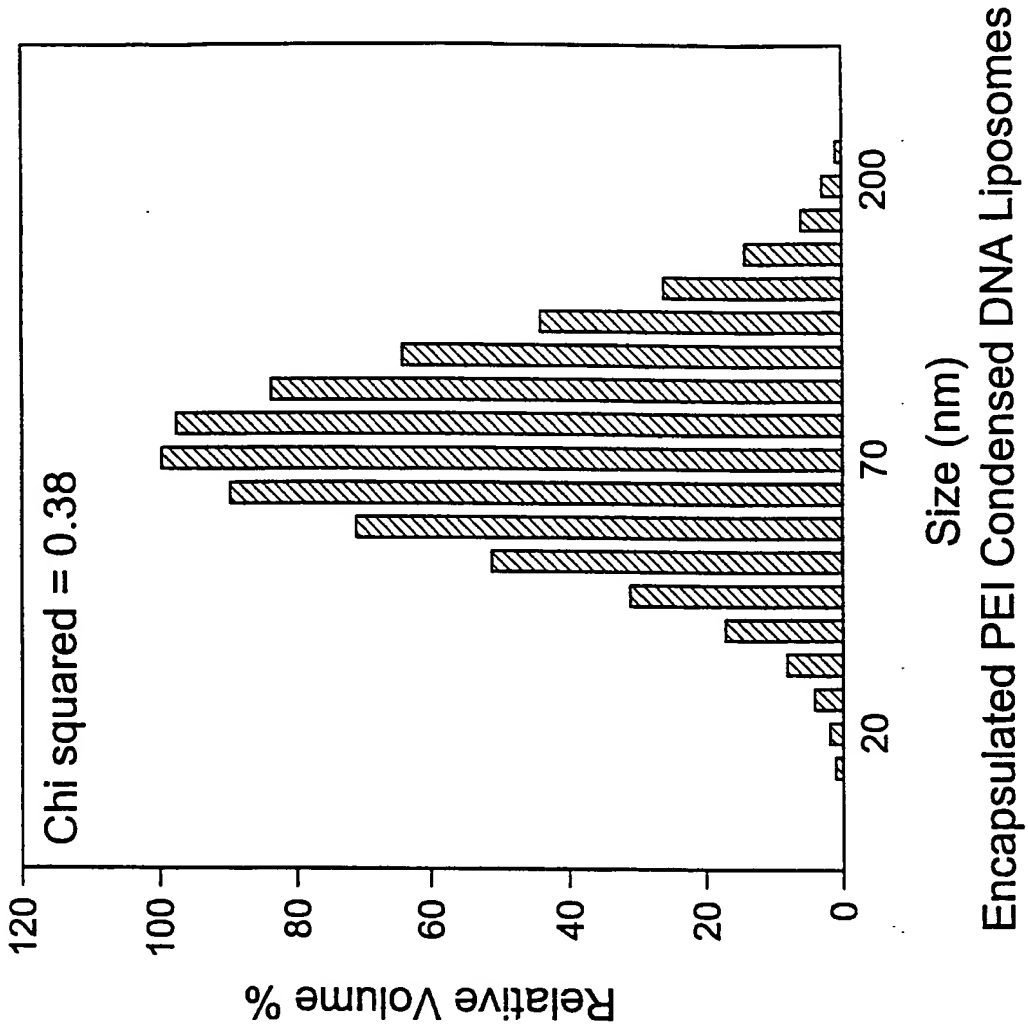

Encapsulated PEI Condensed DNA Liposomes
Chi squared = 0.38
Relative Volume %
Size (nm)
0
20
40
60
80
100
120
20
70
200

Fig. 9

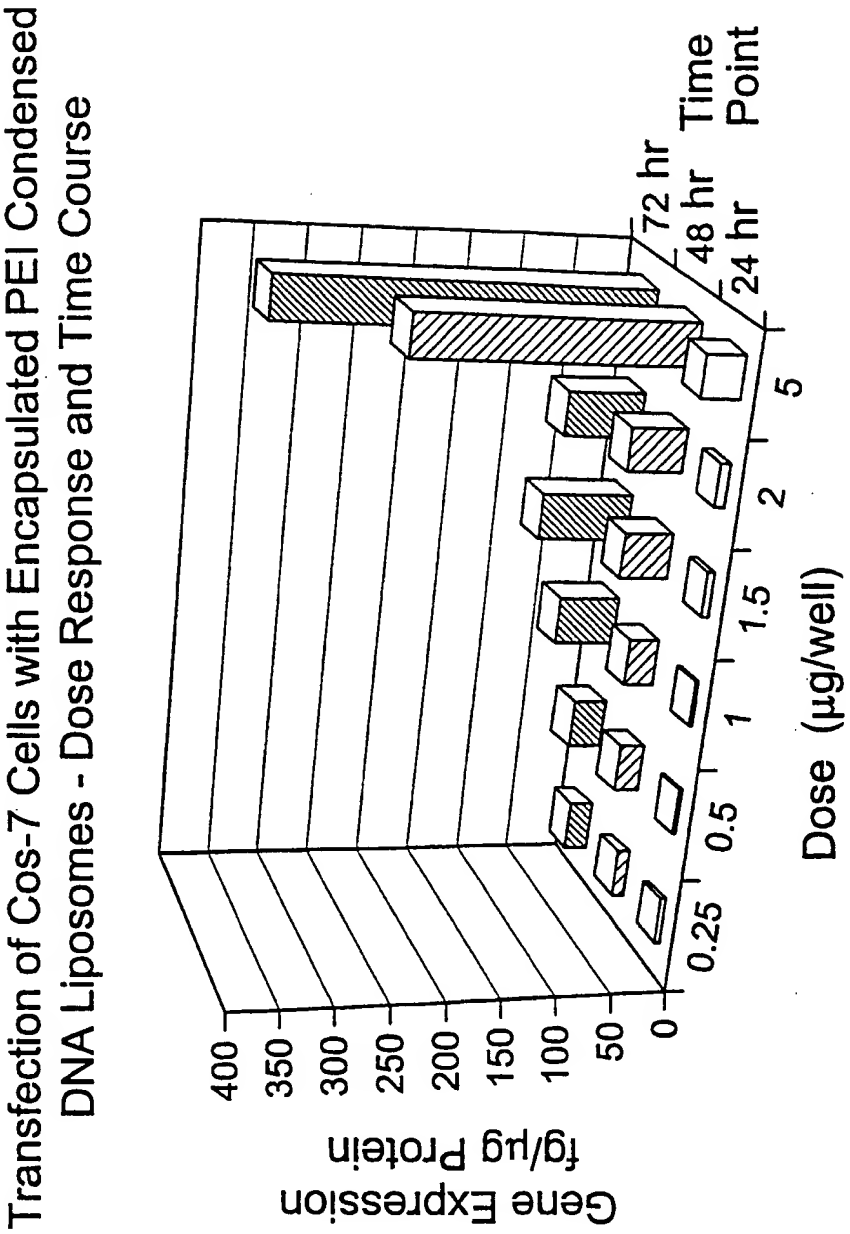

Transfection of Cos-7 Cells with Encapsulated PEI Condensed DNA Liposomes - Dose Response and Time Course
Gene Expression fg/µg Protein
0
50
100
150
200
250
300
350
400
Dose (µg/well)
0.25
0.5
1
1.5
2
5
Time Point
24 hr
48 hr
72 hr

Fig. 10

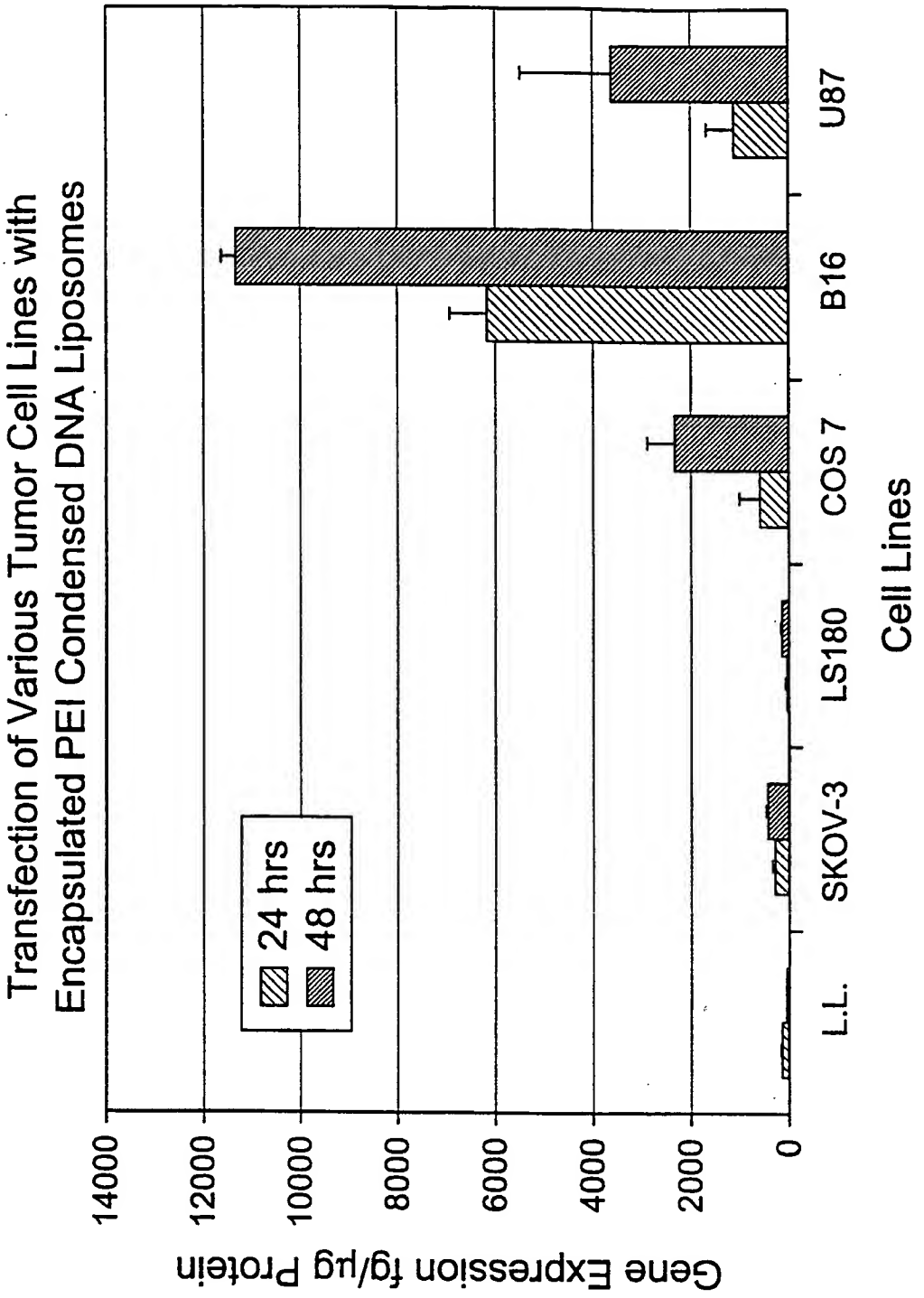

Transfection of Various Tumor Cell Lines with
Encapsulated PEI Condensed DNA Liposomes
Gene Expression fg/µg Protein
14000
12000
10000
8000
6000
4000
2000
0
24 hrs
48 hrs
L.L.
SKOV-3
LS180
COS 7
B16
U87
Cell Lines

Fig. 11

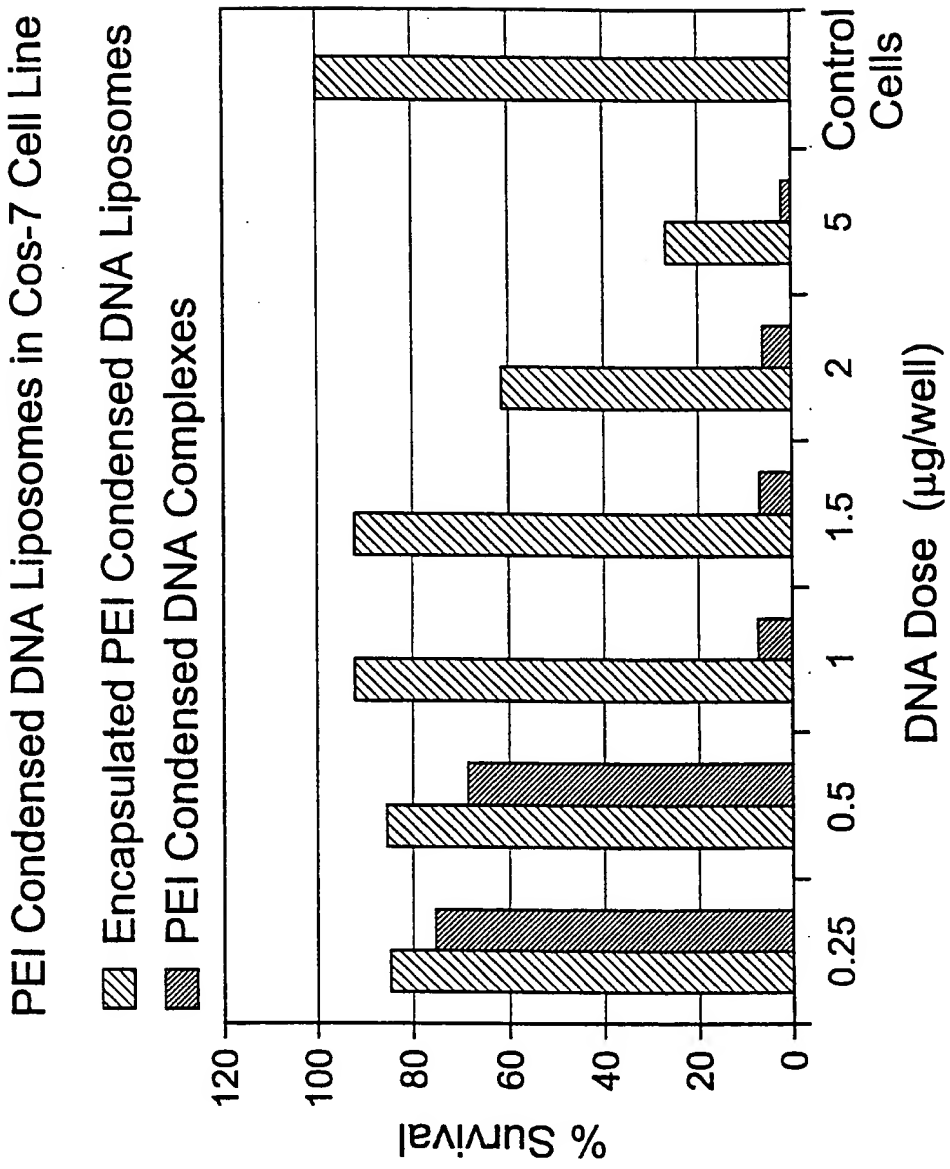

In Vitro Toxicity of Encapsulated
PEI Condensed DNA Liposomes in Cos-7 Cell Line
Encapsulated PEI Condensed DNA Liposomes
PEI Condensed DNA Complexes
% Survival
120
100
80
60
40
20
0
0.25
0.5
1
1.5
2
5
Control Cells
DNA Dose (µg/well)

Fig. 12

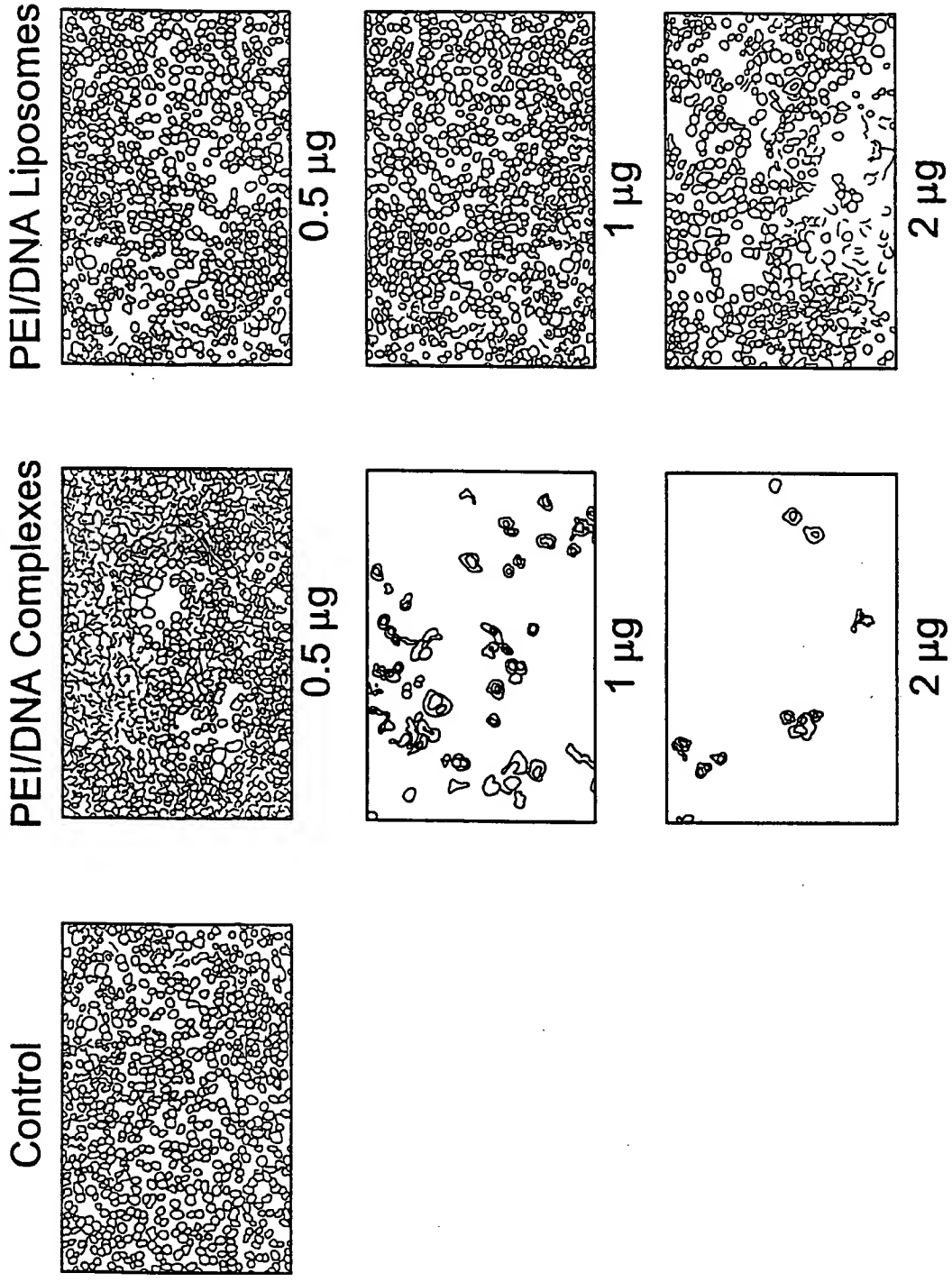

Encapsulated PEI/DNA Liposomes
0.5 µg
1 µg
2 µg
PEI/DNA Complexes
0.5 µg
1 µg
2 µg
Control

Fig. 13

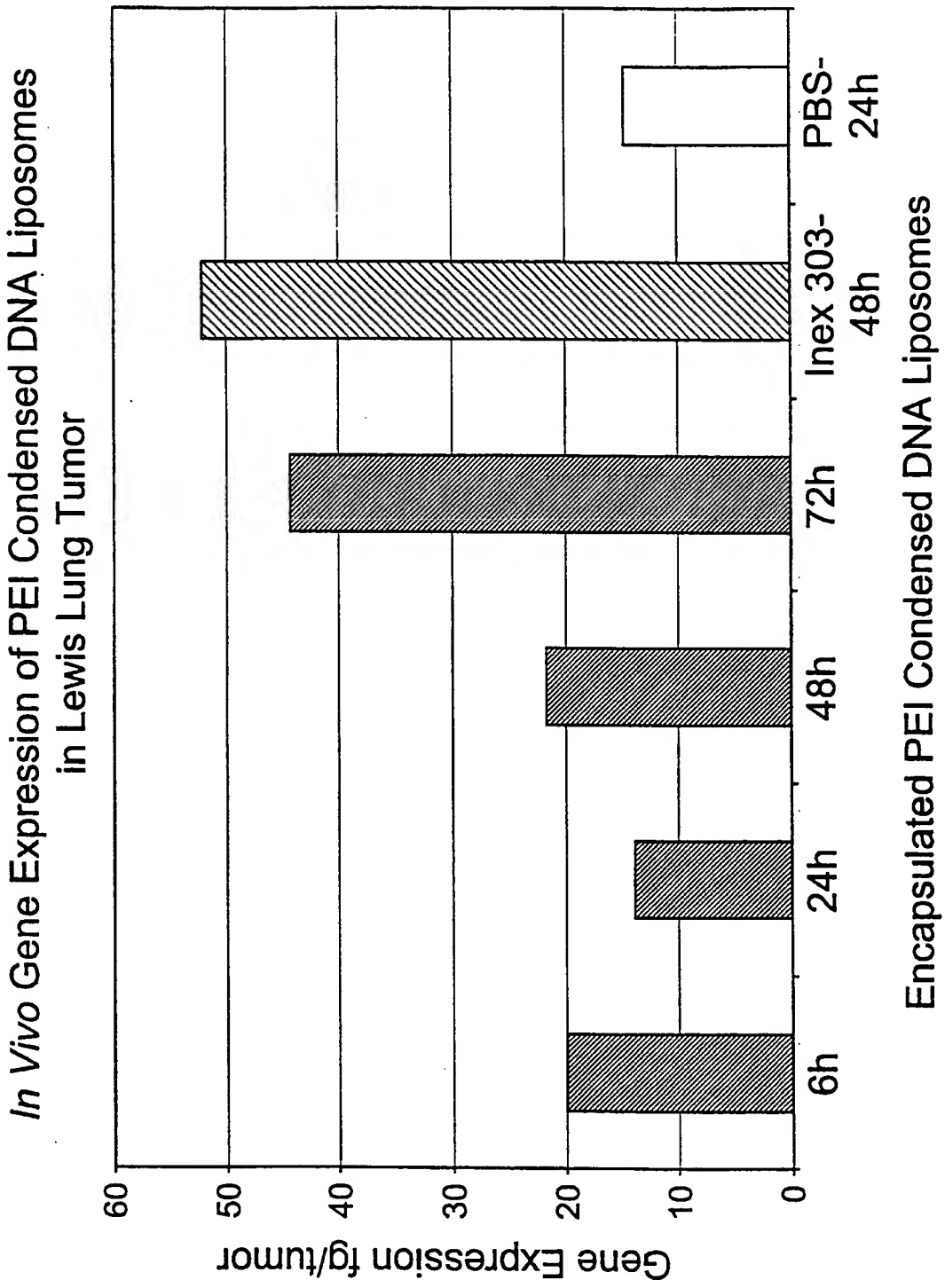

In Vivo Gene Expression of PEI Condensed DNA Liposomes in Lewis Lung Tumor
Gene Expression fg/tumor
60
50
40
30
20
10
0
6h
24h
48h
72h
Inex 303-48h
PBS-24h
Encapsulated PEI Condensed DNA Liposomes

Fig. 14

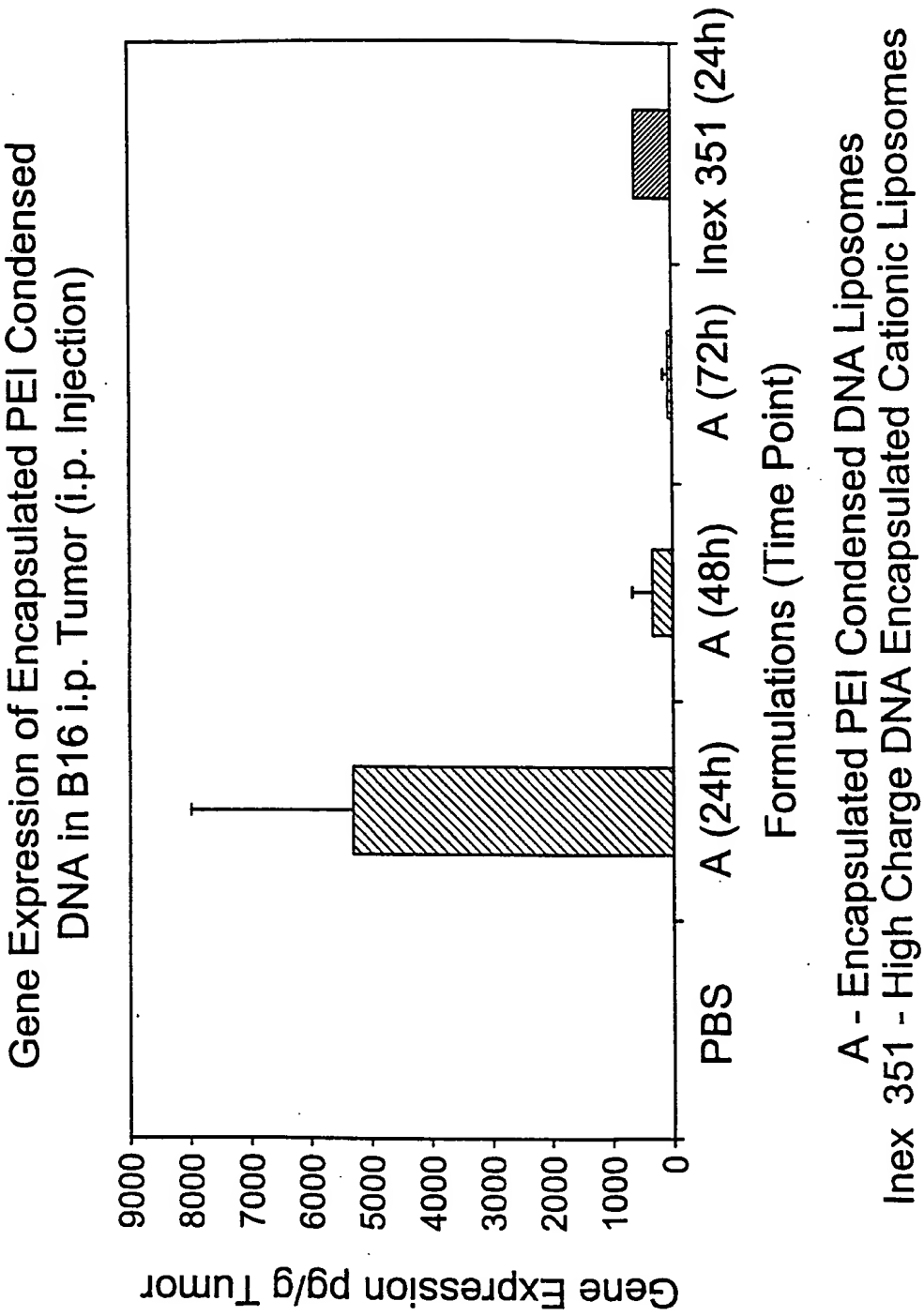
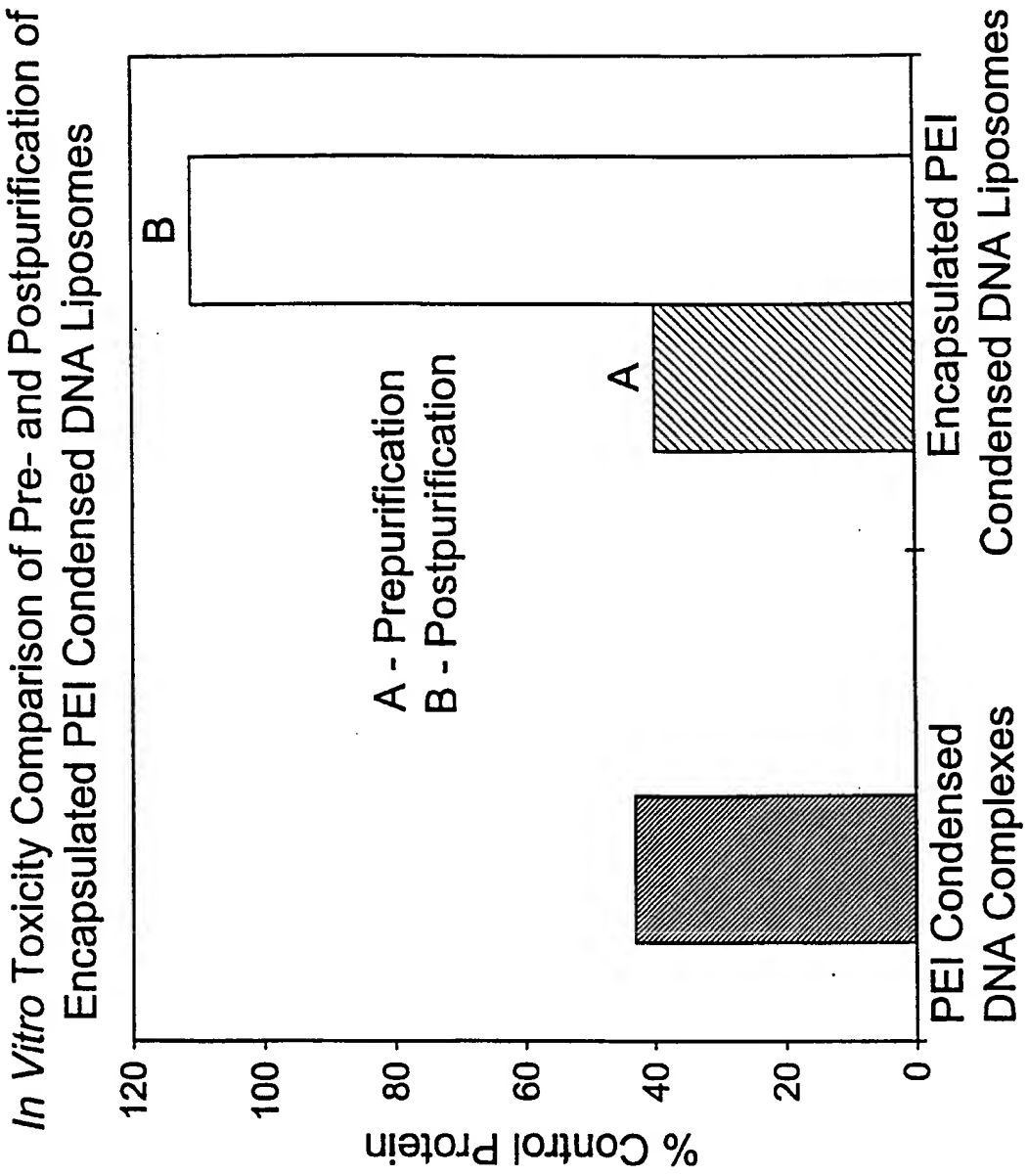

Gene Expression of Encapsulated PEI Condensed DNA in B16 i.p. Tumor (i.p. Injection)
Gene Expression pg/g Tumor
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
PBS
A (24h)
A (48h)
A (72h)
Inex 351 (24h)
Formulations (Time Point)
A - Encapsulated PEI Condensed DNA Liposomes
Inex 351 - High Charge DNA Encapsulated Cationic Liposomes

Fig. 15


In Vitro Toxicity Comparison of Pre- and Postpurification of Encapsulated PEI Condensed DNA Liposomes
% Control Protein
120
100
80
60
40
20
0
A - Prepurification
B - Postpurification
A
B
PEI Condensed DNA Complexes
Encapsulated PEI Condensed DNA Liposomes

PCT ORGANISATION MONDIALE DE LA PROPRIETE INTELLECTUELLE
Bureau international

DEMANDE INTERNATIONALE PUBLIEE EN VERTU DU TRAITE DE COOPERATION EN MATIERE DE BREVETS (PCT)

(51) Classification internationale des brevets 7 : A61K 39/39, 39/145, 39/155, A61P 37/04, 31/12 | A2

(11) Numéro de publication internationale: **WO 00/15256**

(43) Date de publication internationale: 23 mars 2000 (23.03.00)

(21) Numéro de la demande internationale: PCT/FR99/02177

(22) Date de dépôt international: 13 septembre 1999 (13.09.99)

(30) Données relatives à la priorité:
98/11520 11 septembre 1998 (11.09.98) FR

(71) Déposant *(pour tous les Etats désignés sauf US)*: PASTEUR MERIEUX SERUMS ET VACCINS [FR/FR]; 58, avenue Leclerc, F-69007 Lyon (FR).

(72) Inventeur; et

(75) Inventeur/Déposant *(US seulement)*: HAENSLER, Jean [FR/FR]; 17, rue J. Piccandet, F-69290 Saint Genis les Ollières (FR).

(74) Mandataire: KERNEIS, Danièle; Pasteur Mérieux Sérums et Vaccins, Direction de la Propriété Industrielle, 58, avenue Leclerc, F-69007 Lyon (FR).

(81) Etats désignés: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, brevet ARIPO (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), brevet eurasien (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), brevet européen (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), brevet OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Publiée

Sans rapport de recherche internationale, sera republiée dès réception de ce rapport.

(54) Title: IMMUNOSTIMULANT EMULSION

(54) Titre: EMULSION IMMUNOSTIMULANTE

(57) Abstract

The invention concerns an oil-in-water immunostimulant emulsion comprising an aqueous phase and an oil phase, characterised in that it further comprises an immunostimulant poymucleotide whereof at least part is covalently bound to at least a lipid molecule. The invention also concerns a vaccine composition comprising such an emulsion as immunoadjuvant.

(57) Abrégé

La présente invention concerne une émulsion immunostimulante de type huile dans eau, qui comprend une phase aqueuse et une phase huileuse, caractérisée en ce qu'elle comprend en outre au moins un polynucléotide immunostimulant dont au moins une partie est couplée de façon covalente à au moins une molécule lipidique. L'invention concerne également une composition vaccinale comprenant à titre d'immunoadjuvant, une telle émulsion.

UNIQUEMENT A TITRE D'INFORMATION

Codes utilisés pour identifier les Etats parties au PCT, sur les pages de couverture des brochures publiant des demandes internationales en vertu du PCT.

AL	Albanie	ES	Espagne	LS	Lesotho	SI	Slovénie
AM	Arménie	FI	Finlande	LT	Lituanie	SK	Slovaquie
AT	Autriche	FR	France	LU	Luxembourg	SN	Sénégal
AU	Australie	GA	Gabon	LV	Lettonie	SZ	Swaziland
AZ	Azerbaïdjan	GB	Royaume-Uni	MC	Monaco	TD	Tchad
BA	Bosnie-Herzégovine	GE	Géorgie	MD	République de Moldova	TG	Togo
BB	Barbade	GH	Ghana	MG	Madagascar	TJ	Tadjikistan
BE	Belgique	GN	Guinée	MK	Ex-République yougoslave de Macédoine	TM	Turkménistan
BF	Burkina Faso	GR	Grèce	ML	Mali	TR	Turquie
BG	Bulgarie	HU	Hongrie	MN	Mongolie	TT	Trinité-et-Tobago
BJ	Bénin	IE	Irlande	MR	Mauritanie	UA	Ukraine
BR	Brésil	IL	Israël	MW	Malawi	UG	Ouganda
BY	Bélarus	IS	Islande	MX	Mexique	US	Etats-Unis d'Amérique
CA	Canada	IT	Italie	NE	Niger	UZ	Ouzbékistan
CF	République centrafricaine	JP	Japon	NL	Pays-Bas	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norvège	YU	Yougoslavie
CH	Suisse	KG	Kirghizistan	NZ	Nouvelle-Zélande	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	République populaire démocratique de Corée	PL	Pologne		
CM	Cameroun	KR	République de Corée	PT	Portugal		
CN	Chine	KZ	Kazakstan	RO	Roumanie		
CU	Cuba	LC	Sainte-Lucie	RU	Fédération de Russie		
CZ	République tchèque	LI	Liechtenstein	SD	Soudan		
DE	Allemagne	LK	Sri Lanka	SE	Suède		
DK	Danemark	LR	Libéria	SG	Singapour		
EE	Estonie						

EMULSION IMMUNOSTIMULANTE

L'invention concerne le domaine des vaccins et plus particulièrement les adjuvants de vaccins.

Les vaccins, qu'ils soient prophylactiques ou thérapeutiques, sont destinés à stimuler le système immunitaire de l'organisme humain ou animal auquel ils sont administrés, la réponse du système immunitaire pouvant être soit une réponse de type humorale (production d'anticorps), soit une réponse de type cellulaire, soit encore une combinaison des 2 types de réponses. De façon classique, depuis de nombreuses années, la vaccination a consisté à administrer à un organisme une version non pathogène d'un micro-organisme de façon à préparer le système immunitaire à réagir efficacement dans le cas où l'organisme serait amené ultérieurement à rencontrer le même micro-organisme, dans sa version pathogène. L'antigène administré lors de la vaccination peut être de différentes natures : micro-organisme tué entier ou fragmenté, souche vivante atténuée du micro-organisme, fractions antigéniques du micro-organisme ou encore polynucléotides susceptibles de conduire à l'expression par l'organisme d'une fraction antigénique.

Depuis déjà longtemps, on a cherché à augmenter la réponse du système immunitaire ou à modifier sa nature, non pas simplement en agissant sur l'antigène administré ou sur son mode d'administration, mais également en lui adjoignant des substances immunostimulatrices ou adjuvants. Depuis l'adjuvant complet de Freund, de nombreux produits ont été testés, notamment des sels minéraux (tels que le chlorure de zinc, le phosphate de calcium, l'hydroxyde d'aluminium ou encore le phosphate d'aluminium par exemple), des saponines, des polymères, des lipides ou des fractions lipidiques(Lipide A, Monophosphoryl Lipid A),...etc. Cependant, peu d'entre eux présentent toutes les caractéristiques souhaitées : être de bons immuno-adjuvants, stables, mais sans risque de toxicité.

On connaît d'autre part, par la demande WO 96/02555, des oligonucléotides pouvant avoir une activité immunostimulatrice, ces oligonucléotides pouvant être administrés comme adjuvant vaccinal. Cette

référence mentionne également la possibilité d'associer à ces oligonucléotides par liaison ionique, covalente ou par encapsulation, des moyens pour cibler l'administration de l'oligonucléotide. De tels moyens peuvent être notamment constitués de stérol, de lipide (par exemple un lipide cationique, un virosome ou un liposome) ou un agent de liaison spécifique à la cellule-cible (par exemple un liant reconnu par un récepteur spécifique de la cellule-cible). Cette demande mentionne encore, parmi toutes les variantes d'utilisation des polynucléotides décrits, la possibilité de les administrer en conjonction avec un véhicule porteur pharmaceutiquement acceptable. Cette demande n'identifie pas de véhicule comme étant d'un intérêt particulier mais en donne une liste indicative et cite à cet égard notamment les solutions, les solvants, les milieux de dispersion, les agents-retard, les émulsions et autres; l'utilisation de tels milieux pour des substances pharmaceutiquement actives étant mentionnée comme étant bien connue dans ce domaine.

Selon l'enseignement de ce document, la quantité d'oligonucléotides administrée doit l'être en quantité suffisante pour réaliser l'effet biologique recherché.

Or, les auteurs de la présente invention ont trouvé que, de façon tout à fait inattendue, il était possible d'accroître fortement l'effet immunoadjuvant d'un oligonucléotide, sans être obligé d'accroître la quantité d'oligonucléotides ou la quantité d'antigènes administrée.

Pour atteindre ce but, l'invention a pour objet une émulsion immunostimulante du type huile dans eau, comprenant au moins une phase aqueuse et une phase huileuse, caractérisée en ce qu'elle comprend en outre au moins un polynucléotide immunostimulant dont au moins une partie est couplée de façon covalente à au moins une molécule lipidique.

Aux fins de l'invention, on entend par émulsion de type huile dans eau, une dispersions de gouttelettes d'huile dans une phase aqueuse pouvant être constituée par du tampon tel que le tampon PBS. La phase huileuse est constituée par une huile pharmaceutiquement acceptable, qui peut être une huile minérale, animale ou végétale. De préférence, on utilise une huile métabolisable telle que le squalène, les esters (notamment l'oléate d'éthyle, le

myristate d'isopropyle), une huile végétale(par exemple l'huile de ricin, l'huile de tournesol, l'huile d'olive...)ou encore une huile végétale modifiée (ex : les macrogol glycerides). On peut, notamment, obtenir une émulsion satisfaisante en mélangeant 500 mg de squalène à 10 ml de tampon PBS dans un appareil tel qu'un ULTRA-TURRAX™, puis en microfluidisant la dispersion obtenue grâce à un microfluidiseur tel que le Microfluidics™, ce qui permet d'obtenir des particules huileuses dont le diamètre est inférieur à 200 nm.

Afin de faciliter la formation de l'émulsion, il est possible d'utiliser en outre un agent tensio-actif , notamment un agent tensio-actif dont la valeur HLB (Balance Hydrophile/Lipophile) est comprise entre 6 et 14. Il est notamment possible d'utiliser un agent tensio-actif choisi parmi la liste des produits suivants : les esters de sorbitan et les polysorbates, l'huile de ricin éthoxylée hydrogénée ou non, l'acide stéarique éthoxylé, l'alcool oléique 10 OE, l'alcool cétostéarylique 20 OE, le stéarate de glycérol, le stéarate de propylène glycol, les lécithines, le lauryl sulfate de sodium, le stéarate de sodium, le cocoate de glycerol éthoxylé 70E , les esters de glycérol éthoxylés, les acides oléiques éthoxylés, l'oléate de mannitan. On a obtenu de particulièrement bons résultats en utilisant du TWEEN ™ 80.

L'émulsion obtenue est considérée immunostimulante si elle est capable de provoquer ou d'accroître la stimulation du système immunitaire, par exemple lors de son administration conjointement à un antigène vaccinal. Dans cette application, l'émulsion est utilisée comme immuno-adjuvant.

Cette activité immuno-adjuvante peut s'exprimer de différentes façons :

- rendre visible la réponse du système immunitaire à l'administration conjointe de l'antigène et de l'émulsion, alors que la réponse à l'administration de l'antigène seul ne l'était pas,
- accroître le degré de la réponse du système immunitaire sans en modifier la nature (par exemple : augmenter la quantité d'anticorps produits),
- modifier la nature de la réponse du système immunitaire à l'administration de l'antigène (par exemple, induire une réponse cellulaire alors que l'administration de l'antigène seul provoquait uniquement une réponse humorale),

- induire ou accroître la production de cytokines, ou de certaines cytokines en particulier.

Par "polynucléotide" au sens de la présente invention, on comprend un oligonucléotide simple brin ayant de 6 à 100 nucléotides, de préférence de 6 à 30 nucléotides. Il peut s'agir d'oligoribonucléotide, ou d'oligodesoxyribonucléotide. On utilise de préférence des polynucléotides comprenant des séquences de base à symétrie inversée, tel que cela est le cas dans les séquences palindromiques (c'est-à-dire des séquences de type ABCDEE'D'C'B'A' où A et A', B et B', C et C', D et D' E et E' sont des bases complémentaires au sens de Watson et Crick), et plus particulièrement des polynucléotides comprenant au moins une séquence dinucléotidique Cytosine, Guanine, dans laquelle la Cytosine et la Guanine ne sont pas méthylées. Tout autre polynucléotide connu pour être, par sa nature même, immunostimulant, peut convenir aux fins de l'invention. Ainsi, il est possible également d'utiliser les oligonucléotides immunostimulants décrits dans la demande de brevet WO96/02555. On a obtenu de particulièrement bons résultats en utilisant un polynucléotide dont la séquence des bases est la suivante : GAGAACGCTCGACCTTCGAT.

Les oligonucléotides convenant aux fins de l'invention peuvent se présenter sous forme de phosphodiester, ou afin d'être plus stables sous forme de phosphorothioates ou d'hybrides phosphodiester/phosphorothioates. Bien qu'il soit possible d'utiliser des oligonucléotides provenant de sources d'acides nucléiques existantes tel que l'ADN génomique ou le cADN, on préfère utiliser des oligonucléotides de synthèse. Ainsi, il est possible d'élaborer des oligonucléotides sur support solide en utilisant la méthode β-cyano éthyl phosphoramidite (Beaucage, S.L. and Caruthers, M.H. Tetrahedron Letters 22, 1859 - 1862 (1981)) pour l'assemblage 3'-5', puis on procède à une précipitation en éthanol en présence d'acétate de sodium 0,3 M non ajusté en pH (0,3M en final). On effectue ensuite une précipitation par 4 volumes d'éthanol à 80% suivi d'un séchage avant de procéder à une reprise par de l'eau pure.

Les oligonucléotides phosphorothioatés ont un des atomes d'Oxygène composant le groupement Phosphate qui est remplacé par un atome de Soufre. Leur synthèse peut être effectuée comme précédemment décrit, sauf à remplacer la solution iode/eau/pyridine tétrahydrofurane qui est utilisée lors de l'étape d'oxydation nécessaire à la synthèse des liaisons phosphodiester par une solution TETD (tétraethylthiuram disulfide) apportant les ions sulfates permettant de produire le groupement phosphorothioate.

On peut également envisager d'autres modifications des liaisons phosphodiesters, des bases ou des sucres, pour modifier les propriétés des oligonucléotides utilisés, et notamment pour accroître leur stabilité.

Selon l'invention, on couple au polynucléotide au moins une molécule lipidique de façon covalente. Cette molécule lipidique est de préférence une molécule de cholestérol ou de dérivé de cholestérol. Le couplage peut être effectué par liaison covalente à une ou à chaque extrémité du polynucléotide, ou encore par insertion à côté de chaque base d'au moins une molécule lipidique. Ce couplage peut être effectué directement lors de la synthèse du polynucléotide en utilisant dans le synthétiseur d'oligonucléotides un réactif de type Cholestérol Phosphoramidite au lieu du réactif Phosphoramidite habituellement utilisé.

Les antigènes dont il est possible de potentialiser l'effet grâce à l'émulsion selon la présente invention, peuvent être de nature variée ; il peut notamment s'agir de protéines, de glycoprotéines, de glycoconjugués, de polyosides ou encore de polynucléotides comprenant des fractions d'ADN susceptibles de conduire à l'expression de molécules d'intérêt ; il peut également s'agir d'un mélange de différents antigènes. De particulièrement bons résultats ont été obtenus avec une composition comprenant des antigènes de la grippe tels qu'ils sont présents dans le vaccin commercial VAXIGRIP™.

On peut obtenir une émulsion selon l'invention en procédant de la façon suivante : on mélange tout d'abord, sous agitation, l'huile avec la phase aqueuse constituée éventuellement par une solution tampon dans laquelle a été incorporé un surfactant. Le mélange obtenu est homogénéisé au moyen, par exemple, d'un agitateur à hélice, afin de conduire à une émulsion du type

huile dans eau. De préférence, on traite ensuite l'émulsion obtenue au moyen d'un microfluidiseur afin de réduire les gouttelettes d'huile à un diamètre inférieur à 200 nm.

Puis, cette émulsion étant maintenue sous agitation, on lui ajoute simplement le polynucléotide auquel a été couplé le lipide, et on obtient l'émulsion objet de la présente invention.

Lorsque cette émulsion est destinée à être utilisée comme immuno-adjuvant, on la mélange sous agitation, à une composition comprenant l'antigène dont on souhaite potentialiser l'effet. Le mélange peut être avantageusement effectué dans un rapport volumique de 1. On peut, ensuite, vérifier l'effet inattendu et notamment l'effet synergique obtenu sur la stimulation du système immunitaire par l'utilisation simultanée d'un polynucléotide couplé à au moins une molécule de lipide, et son incorporation à une émulsion de type huile dans eau.

A cette fin, il est possible de réaliser un test d'immunogénicité sur des souris divisées en plusieurs groupes, à qui on administre, suivant le groupe :

- soit une composition comprenant uniquement l'antigène ou le mélange d'antigènes vis-à-vis desquels on veut tester l'effet immunostimulant de l'émulsion selon l'invention,
- soit une composition, comprenant l'antigène ou les antigènes d'intérêt à laquelle a été ajoutée une solution comprenant uniquement des polynucléotides couplés à au moins une molécule de lipides,
- soit une composition comprenant l'antigène ou les antigènes d'intérêt à laquelle a été ajoutée une émulsion de type huile dans eau, sans polynucléotide, ou avec un polynucleotide dépourvu d'activité immunostimulante vis-à-vis des antigènes administrés,
- soit une composition comprenant l'antigène ou les antigènes d'intérêt à laquelle a été ajoutée une émulsion selon l'invention.

Pour chacune des souris immunisées, on peut, ensuite, déterminer la quantité et la nature des anticorps produits ce qui permet de déterminer la GMT (ou Moyenne Géométrique du Titre en Anticorps) ; on peut également effectuer des dosages des cytokines produites ; en outre, on peut effectuer des

dosages permettant de déterminer la réponse cellulaire du système immunitaire.

Les résultats obtenus ont montré un effet synergique important des éléments constituant l'émulsion selon l'invention.

De plus, l'émulsion obtenue selon l'invention présente une stabilité accrue par rapport aux émulsions de même nature, i.e. celles constituées d'une phase aqueuse et d'une phase huileuse identiques, mais dépourvues de polynucléotides.

Les exemples qui suivent illustrent de façon plus précise un mode de réalisation de l'invention.

Exemple 1

On prépare des oligonucléotides grâce à un automate synthétiseur fourni par Applied Biosystems qui met en œuvre la méthode chimique standard au phosphoramidite et qui comporte à chaque cycle une étape d'oxydation.

Cette étape d'oxydation est réalisée au moyen d'une solution iode/eau/tétrahydrofurane/acétonitrile pour obtenir une liaison phosphodiester et au moyen d'une solution tétraéthylthiuram/acétonitrile pour obtenir une liaison phosphorothioate. On prépare ainsi un oligonucléotide 3 Db(S) dont la séquence est reproduite sous SEQ ID NO 1 et qui comporte des liaisons phosphorothioate sur toute sa longueur.

On prépare également un oligonucléotide MGC (S) dont la séquence est reproduite à SEQ ID NO 2, qui comporte à la fois des liaisons phosphodiester et des liaisons phosphorothioate. Les liaisons phosphorothiate sont situées à chaque extrémité ; il y a 2 liaisons phosphorothioate en 3' et 5 liaisons phosphorothioate en 5'. Cet oligonucléotide ne possède pas de séquence palindromique, et notamment pas de séquence CG.

Exemple 2

On prépare des oligonucléotides auxquels sont couplés aux extrémités des molécules de cholestérol. La synthèse de ces oligonucléotides 3 Db(S)-chol et

MGC(S)-chol est effectuée de la même manière qu'à l'exemple 1, à l'exception du réactif Phosphoramidite qui est remplacé par un réactif spécifique, le Cholestérol-ON™ Phosphoramidite fourni par la société CLONTECH Lab. Inc, (USA), lors du premier et du dernier cycle de synthèse afin d'obtenir une molécule de cholestérol insérée avant chacun des nucléotides d'extrémité.
Les séquences de nucléotides obtenues sont identiques à celles des oligonucléotides décrits à l'exemple précédent.

Exemple 3

On dispose de 10 ml de tampon PBS auxquels on ajoute 25 mg de Tween™80 et 500 mg de squalène. Le mélange obtenu est émulsionné grâce à un appareil ULTRA-TURRAX™ T25 pendant 1 min à 13500 tours/min.
L'émulsion obtenue est ensuite fluidisée grâce à un traitement de 5 cycles à 500 Psi dans un microfluidiseur Microfluidics™.

Exemple 4

Préparation d'une émulsion squalène/PBS comprenant des polynucléotides couplés à du cholestérol.
On prépare une émulsion immunostimulante selon l'invention en mélangeant 435 µl de la solution à 2,3 g/l de 3Db(S) couplé au cholestérol obtenue à l'exemple 2 (soit 1 mg d'oligonucléotide), avec 2 ml de l'émulsion squalène/PBS obtenue à l'exemple 3, maintenue sous agitation.
On prépare une autre émulsion en mélangeant 263 µl de la solution à 3,81 g/l de MGC(S) couplé à du cholestérol obtenue à l'exemple 2 (soit 1 mg d'oligonucléotide) avec 2 ml de l'émulsion squalène/PBS obtenue à l'exemple 3, maintenue sous agitation.

Exemple 5

Préparation des compositions d'immunisation.

On prépare des doses d'immunisation de différentes natures en ajoutant sous agitation 2 ml de vaccin splitté contre la grippe NIB16 (monovalent A/Singapore H1N1) contenant 100 µg d'hémagglutinine HA en tampon PBS à 2 ml de chacune des préparations suivantes :

- tampon PBS
- solution MGC(S) obtenue à l'exemple 1,
- solution MGC(S)-chol obtenue à l'exemple 2,
- émulsion MGC(S)-chol obtenue à l'exemple 4,
- solution 3Db(S) obtenue à l'exemple 1,
- solution 3Db(S)-chol obtenue à l'exemple 2,
- émulsion 3Db(S)-chol obtenue à l'exemple 4.

Exemple 6

Immunisation.

On dispose de groupes de 6 souris Balb/c femelles âgées de 6 à 8 semaines, chaque groupe correspondant à une des préparations effectuées à l'exemple 6. Chacune des souris est immunisée avec 200µl de la préparation correspondant à son groupe et reçoit donc 5µg de HA par immunisation, chaque souris étant immunisée 2 fois à 3 semaines d'intervalle, avec la même préparation.

2 semaines après la deuxième injection, on mesure la réponse en anticorps spécifiques anti-HA, grâce à un test ELISA, et on détermine la GMT pour les IgG1 ainsi que pour les IgG2a.

Les résultats obtenus sont indiqués ci-après :

	GMT/IgG1	GMT/IgG2a
HA uniquement	38 062	2 137
HA/MGC(S)	37 518	1 050
HA/MGC(S)-chol	28 039	1 498
HA/MGC(S)-chol/émulsion	264 776	26 981
HA/3Db(S)	63 939	43 529
HA/3Db(S)-chol	65 904	31 066
HA/3Db(S)-chol/émulsion	611 301	218 142

Les résultats obtenus confirment que l'oligonucléotide 3Db(S) est bien doté de propriétés immunostimulantes car il est capable d'induire un accroissement de la réponse en anticorps par rapport à ce qui est obtenu lors de l'administration des antigènes seuls. Par contre, les résultats obtenus avec l'oligonucléotide MGC(S) ne démontrent pas d'activité immunostimulante .

D'autre part, on remarque que, de façon inattendue, l'émulsion contenant un polynucléotide immunostimulant tel que le polynucléotide 3Db(S) conduit à une production d'anticorps nettement supérieure à celle obtenue avec une émulsion contenant le polynucléotide MGC(S)-chol; cet effet est encore plus remarquable en ce qui concerne la production d'IgG2a; ce qui est indicateur d'une orientation de la réponse immunitaire vers un type TH1, orientation parfois souhaitée dans certaines cibles vaccinales.

En effet, en considérant que l'effet attendu d'une émulsion est l'effet obtenu avec l'émulsion HA/MGC(S)-chol, (l'oligonucléotide MGC(S) n'ayant par lui-même aucun effet immunostimulant vis-à-vis des antigènes administrés ainsi que cela est analysé ci-dessus), on note un effet synergique important de l'émulsion selon l'invention car le titre obtenu pour la production d'anticorps, que ce soit pour les IgG1 ou de façon plus nette encore pour les IgG2a, est nettement supérieur à la somme des titres obtenus séparément pour chacune des 2 compositions (émulsion HA/MGC(S) d'une part et solution HA/3Db(S) d'autre part)

Exemple 7.

On prépare des compositions vaccinales comprenant les éléments suivants:

- antigènes sous-unitaires contre le RSV (ou Virus Syncitial Respiratoire) en présence de gel d'aluminium, à raison de 1 µgramme de protéines totales (Protéines F, G et M)

en tampon PBS ou additionné suivant le cas des éléments suivants:

- solution 3Db(S) obtenue à l'exemple 1,
- émulsion 3 Db(S) obtenue à l'exemple 4,
- émulsion MGC(S) obtenue à l'exemple 4.

Les doses sont de 50µlitres et comprennent 50 microgrammes d'oligonucléotides.

Ces compositions sont administrées à des souris à J0 et à J28; 5 à 6 semaines après l'injection de rappel, on prélève les rates des souris afin d'évaluer la quantité d'interféron γ produite.

On obtient les résultats suivants, après dosage ELISA effectué après restimulation secondaire in vitro:

	Quantité d' Interféron en pg/ml
Antigènes + Adjuvant aluminium	3432 2565 2998
Antigènes + Adjuvant aluminium + 3 Db(S)	13400 9543 1147
Antigènes + Adjuvant aluminium + émulsion MGC(S)	5130 9216 7173
Antigènes + Adjuvant aluminium + émulsion selon l'invention	57394 42285 49839

Ces résultats montrent clairement la synergie obtenue en utilisant un oligonucléotide immunostimulant et une émulsion selon l'invention , lorsqu'on s'intéresse au RSV et qu'on observe la production d'interféron γ qui est un bon indicateur de la réponse TH1.

Exemple 8:

On prépare des doses d'immunisation identiques à celles de l'exemple 7, à l'exception des antigènes RSV qui ne sont pas en présence de gel d'aluminium. Les doses de 50µlitres sont administrées en intramusculaire à des groupes de 6 souris.

4 semaines après l'immunisation, les souris sont saignées et les taux d'anticorps anti-protéines F sont déterminés par titrage ELISA. Les résultats obtenus sont récapitulés dans le tableau suivant :

	Ig G	Ig G1	Ig G 2a
Antigènes + PBS	100	100	100
	100	100	100
	100	100	100
	100	100	100
	100	100	100
Antigènes +	6400	400	6400
3Db(S)	6400	400	12800
	12800	800	6400
	6400	400	3200
	3200	100	25600
	6400	400	25600
Antigènes +	12800	1600	6400
émulsion MGC(S)	6400	400	100
	51200	6400	100
	25600	1600	100
	25600	1600	100
Antigènes +	25600	1600	25600
émulsion selon	12800	400	12800
l'invention	51200	1600	25600
	102400	6400	1600
	51200	3200	25600

Ces résultats confirment l'intérêt d'utiliser une émulsion selon l'invention dans le cas ou les antigènes sont les antigènes du Virus Syncitial Respiratoire.

REVENDICATIONS

1. Emulsion immunostimulante du type huile dans eau, comprenant au moins une phase aqueuse et une phase huileuse, caractérisée en ce qu'elle comprend en outre au moins un polynucléotide immunostimulant dont au moins une partie est couplée de façon covalente à au moins une molécule lipidique.

2. Emulsion selon la revendication 1, caractérisée en ce que la molécule lipidique est une molécule de cholestérol.

3. Emulsion selon une des revendications précédentes, caractérisée en ce que le polynucléotide immunostimulant comprend au moins une séquence palindromique.

4. Emulsion selon une des revendications précédentes, caractérisée en ce que le polynucléotide immunostimulant est un oligodesoxynucléotide phosphodiester, phosphorothioate ou un hybride phosphodiester phosphorothioate.

5. Emulsion selon une des revendications précédentes, caractérisée en ce que le polynucléotide immunostimulant comprend une séquence GAGAACGCTCGACCTTCGAT.

6. Emulsion selon une des revendications précédentes caractérisée en ce que la partie couplée à au moins une molécule lipidique est située à l'extrémité 5' du polynucléotide.

7. Emulsion selon une des revendications précédentes, caractérisée en ce que la molécule lipidique est une molécule de cholestérol.

8. Emulsion selon une des revendications précédentes, caractérisée en ce qu'elle comprend, en outre, au moins un surfactant.

9. Emulsion selon la revendication 8, caractérisée en ce que le surfactant est du Tween 80.

10. Emulsion selon une des revendications 1 à 9, caractérisée en ce que la phase huileuse comprend du squalène

11. Composition vaccinale comprenant au moins un antigène vaccinal, caractérisée en ce qu'elle comprend en outre une émulsion immunostimulante selon une des revendications 1 à 10.

12. Composition vaccinale selon la revendication précédente, caractérisée en ce qu'elle comprend au moins un antigène vaccinal contre la grippe.

13. Composition vaccinale selon la revendication 11, caractérisée en ce qu'elle comprend au moins un antigène vaccinal du Virus Syncitial Respiratoire.

14. Utilisation d'une émulsion selon une des revendications 1 à 10, pour la fabrication d'un médicament destiné à stimuler le système immunitaire.

15. Utilisation d'une émulsion selon une des revendications 1 à 10, pour la fabrication d'un médicament destiné à produire une réponse de type TH1.

16. Utilisation d'une émulsion selon une des revendications 1 à 10, pour la fabrication d'un médicament destiné à induire la sécrétion d'interféron γ.

LISTE DE SEQUENCES

(1) INFORMATIONS GENERALES:

(i) DEPOSANT:
(A) NOM: Pasteur Merieux Serums & Vaccins
(B) RUE: 58 avenue Leclerc
(C) VILLE: Lyon
(E) PAYS: France
(F) CODE POSTAL: 69007
(G) TELEPHONE: 33 (0) 4 72 73 70 90
(H) TELECOPIE: 33 (0) 4 72 73 78 50

(ii) TITRE DE L' INVENTION: Emulsion immunostimulante

(iii) NOMBRE DE SEQUENCES: 2

(iv) FORME DECHIFFRABLE PAR ORDINATEUR:
(A) TYPE DE SUPPORT: Floppy disk
(B) ORDINATEUR: IBM PC compatible
(C) SYSTEME D' EXPLOITATION: PC-DOS/MS-DOS
(D) LOGICIEL: PatentIn Release #1.0, Version #1.30 (OEB)

(2) INFORMATIONS POUR LA SEQ ID NO: 1:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 20 paires de bases
(B) TYPE: nucl,otide
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: lin,aire

(ii) TYPE DE MOLECULE: Autre acide nucl,ique

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 1:

GAGAACGCTC GACCTTCGAT 20

(2) INFORMATIONS POUR LA SEQ ID NO: 2:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 20 paires de bases
(B) TYPE: nucl,otide
(C) NOMBRE DE BRINS: simple
(D) CONFIGURATION: lin,aire

(ii) TYPE DE MOLECULE: Autre acide nucl,ique

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 2:

GGGGTCAAGC TTGAGGGGGG 20